Developmental Control of Cell Division

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Regulatie van celdeling tijdens de ontwikkeling van meercelligen

(met een samenvatting in het Nederlands)

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Aan mijn vader en moeder

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SAMENVATTING

Voor de ontwikkeling van meercellige organismen is het van belang dat individuele celdelingen gecoördineerd worden met de groei en differentiatie van het hele organisme. Hoewel veel bekend is over de mechanismen die betrokken zijn bij de deling van een cel, is er relatief weinig bekend over hoe signalen van buiten de cel die mechanismen beïnvloeden.

De rondworm *Caenorhabditis elegans* is een uniek diermodel om de mechanismen te identificeren die het samenspel tussen celdeling en ontwikkeling reguleren. Celdelingen kunnen *in vivo* gevolgd worden via lichtmicroscopie. Bovendien is van elke cel het tijdstip van deling en de bestemming van de dochtercellen bekend. Een ander voordeel van *C. elegans* is dat er veel minder kans is op overlappende functies van meerdere genen in één familie. Zo codeert het genoom van *C. elegans* slechts voor één homoloog van de retinoblastoma (Rb), Cycline D en CDK4/6 kinase families. Een laatste voordeel is de beschikbaarheid van krachtige genetische technieken om de functies van genen in *C. elegans* te bestuderen. Hiertoe behoren ondermeer de identificatie van nieuwe genen in "screens", het inactiveren van al bekende genen via de RNA interferentie (RNAi) methode en het isoleren van chemisch geïnduceerde deletie-allelen van specifieke genen met behulp van de polymerasekettingreactie (PCR).

Alvorens *C. elegans* te kunnen gebruiken om de regulatie van celdelingen tijdens de ontwikkeling te bestuderen, moesten eerst de basale mechanismen die nodig zijn voor celdeling in *C. elegans* worden opgehelderd. In hoofdstukken 2 en 3 zijn de resultaten van mijn onderzoek naar diverse regulatoren van de celcyclus in *C. elegans* beschreven. Aangetoond is dat hun functies sterk overeenkomen met die van hun tegenhangers in zoogdieren. De resultaten vormen de basis voor de genetische experimenten beschreven in hoofdstukken 4 en 5, die de identificatie van nieuwe regulatoren van de G_1 fase van de celcyclus tot doel hadden. Hieronder volgt een korte beschrijving van de inhoud van elk hoofdstuk in dit proefschrift.

Hoofdstuk 1 is een algemene introductie van de mechanismen die de deling van een cel bewerkstelligen, met nadruk op de regulatoren van de G_1 fase. In de tweede helft van dit hoofdstuk worden de functies in diermodellen van Cycline D-CDK4/6 kinases, de Rb familie en E2Fs bediscussieerd.

Hoofdstuk 2 beschrijft het onderzoek naar de functie van het cycline afhankelijke kinase *ncc-1*. Het gen dat codeert voor *ncc-1* is nauw verwant aan de humane genen *CDK1/Cdc2, CDK2* en *CDK3*, en de gist genen *CDC28* en *cdc2*$^{+}$. Het onderzoek toont aan dat *ncc-1* een gelijksoortige rol speelt als *CDK1* en *CDC28/cdc2*$^{+}$, en nodig is voor M fase tijdens zowel meiose als mitose. Omdat *ncc-1* niet nodig is voor de G_1/S overgang, is het waarschijnlijk dat *C. elegans* meerdere CDK's gebruikt om de voortgang door de celcyclus te bewerkstelligen, net als andere meercellige organismen. In verdere genetische *screens* identificeerden we inderdaad een CDK die specifiek tijdens de G_1 fase functioneert (zie volgende paragraaf).

In Hoofdstuk 3 is het onderzoek naar de rol van de Cycline D-CDK4/6 - pRb route tijdens de ontwikkeling van *C. elegans* beschreven. In een genetische *screen* isoleerden we mutante allelen van *cyd-1* Cycline D en *cdk-4* CDK4/6, respectievelijk de enige Cycline D en CDK4/6 kinase in *C. elegans*. Inactivatie van *cyd-1* of *cdk-4* resulteerde in blokkade van de larvale celdelingen tijdens de G_1 fase, terwijl de groei van cellen doorging tot lang na het tijdstip waarop de celdeling stagneerde. In *C. elegans* zijn *cyd-1* en *cdk-4* dus voornamelijk nodig voor de voortgang door de G_1 fase.

Om te bepalen of inactivatie van leden van de retinoblastomafamilie de enige functie is van *cyd-1* en *cdk-4* hebben we dubbelmutanten gecreëerd, waarin zowel de retinoblastomafamilie als Cycline D-CDK4/6 kinases volledig zijn geïnactiveerd. Inactivatie van *lin-35*, het enige Rb gen in *C. elegans*, onderdrukte de celdelingsdefecten in *cyd-1* en *cdk-4* mutanten. Dit gaf aan dat *lin-35* Rb een belangrijke negatieve regulator van de G_1/S overgang is. Bovendien toonde dit voor het eerst aan dat *in vivo*, de Cycline D-CDK4/6 kinases een essentiële rol spelen bij het tegengaan van de celdeling remmende werking van de pRb familie.

In zoogdieren maken CDK-remmers van de Cip/Kip familie deel uit van een tweede route die de voortgang door de G_1 fase reguleert. Daarom vergeleken we de rol van *cki-1* Cip/Kip in G_1 regulatie met die van *lin-35* Rb. Wij demonstreerden dat *cki-1* Cip/Kip snelheidsbeperkend is voor de G_1/S overgang, en dat *lin-35* Rb en *cki-1* Cip/Kip allebei bijdragen aan de regulatie van de G_1 fase.

In Hoofdstuk 4 is het onderzoek beschreven naar de mechanismen waarmee *lin-35* Rb voortgang door de G_1 fase remt. In eerste instantie was *lin-35* Rb geïdentificeerd als één van de synthetische multivulva (synMuv) genen, die betrokken zijn bij de vorming van de cellen van de vulva. Om genen te vinden die samen met *lin-35* de voortgang door de G_1 fase reguleren, onderzochten we of andere synMuv genen betrokken zijn bij de regulatie van de G_1 fase. We vonden dat de E2F-homoloog *efl-1* de voortgang door de G_1 fase remt, terwijl de DP homoloog *dpl-1* eigenschappen heeft van zowel een positieve als een negatieve regulator

van de G_1 fase. Waarschijnlijk blokkeren EFL-1 en DPL-1 in een complex met LIN-35 de transcriptie van genen, terwijl DPL-1 met een tweede E2F als activator van transcriptie zou kunnen werken. Dit model komt overeen met de functies die toegeschreven worden aan E2Fs in *Drosophila* en zoogdieren, en ondersteunt de theorie dat in meercellige organismen E2Fs twee tegengestelde functies hebben.

Naast *efl-1* en *dpl-1* identificeerden we *lin-9, lin-15*B en *lin-36* als nieuwe remmers van de G_1 fase. De celcyclus fenotypen van *lin-35* Rb en *lin-36* komen sterk overeen, wat erop duidt dat *lin-35* Rb en *lin-36* binnen dezelfde route voor G_1 regulatie functioneren. Deze resultaten wijzen op een algemene rol voor een deel van de synMuv genen binnen de regulatie van de G_1 fase. Dit soort studies kan leiden tot de identificatie van nieuwe bindingspartners voor *lin-35* Rb, met homologen in zoogdieren die mogelijkerwijs tumorsuppressors zijn.

Tenslotte worden in Hoofdstuk 5 twee projecten beschreven die op dit moment gaande zijn. Inactivatie van *lin-35* Rb onderdrukt de celcyclus defecten in *cyd-1* en *cdk-4* mutanten niet volledig, wat aangeeft dat Cycline D-CDK4/6 kinases nog andere doelwitten hebben naast de pRb familie. Om deze doelwitten te identificeren voerden we een *screen* uit. We isoleerden een interessante mutatie die in combinatie met inactivatie van *lin-35* Rb resulteert in vruchtbare *cyd-1* mutanten. Deze mutatie ligt op het X chromosoom, en we zijn bezig om het gen te identificeren dat door deze mutatie is aangedaan. Dit onderzoek openbaart waarschijnlijk een tweede functie van Cycline D-CDK4/6 kinases die essentieel is *in vivo*.

Het tweede project is gericht op de identificatie van genen die samenwerken met *lin-35* Rb tijdens de ontwikkeling van *C. elegans*. In een proef-*screen* zijn al mutaties gevonden waarvan het fenotype onderdrukt wordt door inactivatie van *lin-35* Rb. Ook is een mutatie gevonden die zelf levensvatbaar is maar in combinatie met inactivatie van *lin-35* Rb een steriel fenotype veroorzaakt. De genen die door deze mutaties aangedaan zijn, kunnen betrokken zijn bij de regulatie van de G_1 fase, of samenwerken met *lin-35* op andere gebieden van de ontwikkeling (op gelijksoortige wijze als de synMuv klasse A genen).

Samenvattend hebben de in dit proefschrift beschreven experimenten drie hoofddoelstellingen bereikt. Ten eerste hebben ze bijgedragen aan het definiëren van de basale celcyclusmachinerie in *C. elegans*. Daarnaast is aangetoond dat voor de regulatie van de celcyclus in *C. elegans* gelijksoortige mechanismen gebruikt worden als in zoogdieren. Deze basis is tenslotte gebruikt om genetische *screens* uit te voeren om nog onbekende genen te vinden die een rol spelen bij de regulatie van de celdeling tijdens de ontwikkeling van *C. elegans*.

SUMMARY

During development of multicellular organisms, cell divisions need to be coordinated with the developmental program of the entire organism. Although the mechanisms that drive cells through the division cycle are well understood, very little is known about the pathways that link extracellular signals to the cell-intrinsic cell-cycle machinery. *C. elegans* provides a unique animal model in which developmental controls of cell-division can be identified genetically. Cell divisions can be followed *in vivo* by light microscopy, and the nearly invariant cell-lineage of *C. elegans* has been completely described. In addition, redundancy between genes is limited in *C. elegans*. For example, the *C. elegans* genome encodes only single members of the retinoblastoma (Rb), Cyclin D and CDK4/6 kinase families. Finally, powerful genetic techniques are available in *C. elegans* to study gene function. These include classic genetic screens, as well as reverse genetic approaches such as RNA-mediated interference and the isolation of chemically induced deletion alleles by PCR.

To use *C. elegans* as a model system to identify developmental regulators of cell-cycle progression, we first needed to identify the basic cell-cycle machinery in *C. elegans*. In Chapters 2 and 3 I describe several cell-cycle regulators in *C. elegans* and demonstrate that they act highly similarly to their mammalian counterparts. These results form the basis for subsequent genetic studies described in Chapters 4 and 5, which were aimed at identifying novel regulators of G_1 progression. Below follows a brief description of the contents of each chapter in this thesis.

In Chapter 1, the general mechanisms that control cell-cycle progression are introduced, with emphasis on G_1 regulators. The second half of the chapter discusses the functions of Cyclin D-CDK4/6 kinases, the Rb family and E2F/DP transcription factors in animal models.

In Chapter 2, we addressed the functions of the cyclin dependent kinase *ncc-1* in *C. elegans*. *ncc-1* is closely related to human *CDK1/Cdc2, CDK2* and *CDK3,* and yeast *CDC28/cdc2*$^+$. We demonstrated that *ncc-1* acts analogously to *CDK1* and *CDC28/cdc2*$^+$, and is specifically required for progression through M phase in meiotic as well as mitotic cell cycles. As *ncc-1* does not appear to be required for the G_1/S transition, *C. elegans* likely uses multiple CDKs to regulate progression through the cell cycle, similar to other metazoans. Indeed, we did identify another CDK with G_1 specific functions in subsequent genetic screens (see below).

In Chapter 3, we examined the function of the Cyclin D-CDK4/6 – pRb pathway in *C. elegans*. In a genetic screen, we identified mutations in *cyd-1* cyclin D and *cdk-4* CDK4/6, the single D-type cyclin and CDK4/6 kinase in *C. elegans,* respectively. Loss of *cyd-1* or *cdk-4* arrested postembryonic cell divisions in G_1 phase, but cell growth continues well beyond the time point of arrest. Thus, *C. elegans cyd-1* and *cdk-4* primarily control G_1 progression.

To determine whether *cyd-1* and *cdk-4* solely act to overcome G_1 inhibition by pRb family members, we constructed double mutants that completely eliminate the function of the pRb family and cyclin D-CDK4/6 kinases. Inactivation of *lin-35* Rb, the single Rb-related gene in *C. elegans*, substantially reduced the cell-division defects in *cyd-1* and *cdk-4* mutant animals. This shows that *lin-35* Rb is an important negative regulator of G_1 progression. Moreover, this is the first *in vivo* demonstration of an essential role for Cyclin D-CDK4/6 kinases in counteracting cell-cycle inhibition by proteins of the pRb family.

In mammals, the Cip/Kip family of CDK inhibitors provides an additional level of control over G1 progression. Therefore, we compared the functions of *lin-35* Rb and *cki-1* Cip/Kip in G_1 regulation. We demonstrated that *cki-1* Cip/Kip, rather than *lin-35* Rb, is rate limiting for G_1/S progression, and that *lin-35* Rb and *cki-1* Cip/Kip contribute nonoverlapping levels of G_1/S control in *C. elegans*.

In Chapter 4 we examined the mechanism of negative regulation of G_1 progression by *lin-35* Rb. *lin-35* Rb was originally identified as a member of the synthetic Multivulva genes, which regulate vulval cell fate specification. To identify genes that act with Rb in regulating G_1 progression, we examined the synthetic Multivulva genes for a role in G_1 regulation. We found that the E2F homolog *efl-1* negatively regulates cell-cycle entry, while the DP related gene *dpl-1* acts both as a positive and negative regulator of G_1 progression. EFL-1 and DPL-1 likely act as transcriptional inhibitors in a complex with LIN-35, while DPL-1 and a second E2F may act as transcriptional activators. This model is in agreement with the proposed functions of E2Fs in *Drosophila* and mammals, and provides further evidence that two opposing E2F functions are present *in vivo*.

In addition to *efl-1* and *dpl-1*, we identified a role for *lin-9*, *lin-15*B and *lin-36* as novel negative regulators of G_1 progression. *lin-35* Rb and *lin-36* showed very similar cell-cycle phenotypes, consistent with a model in which these genes act together in G_1 regulation. These results indicated a role for a subset of the class B synMuv genes in the general regulation of G_1 progression. Some of these genes may be obligatory partners of *lin-35* Rb, and their mammalian counterparts may be candidate tumor suppressors.

Finally, Chapter 5 describes two projects that are currently ongoing. Loss of *lin-35* Rb did not fully suppress the cell-cycle arrest of *cyd*-1 and *cdk-4* mutants, which indicates that Cyclin D-CDK4/6 kinases have targets outside the pRb family. Therefore, we performed a screen to identify such targets. We isolated an interesting mutation that, when combined with inactivation of *lin-35* Rb, confers viability to *cyd-1* mutants. The mutation maps to the X chromosome and we are currently identifying the gene affected. These studies are likely to reveal a second function of Cyclin D-CDK4/6 kinases that is essential *in vivo*.

The second project is aimed at identifying genes that cooperate with *lin-35* Rb during development of *C. elegans*. In a small-scale pilot version of this screen, we already identified mutations whose phenotypes are suppressed by loss of *lin-35*, as well as a mutation that is viable by itself, but causes sterility in combination with inactivation of *lin-35*. These mutations may define genes that act in G_1 regulation, either upstream of or in parallel to *lin-35*, and genes that interact with *lin-35* Rb in other developmental processes (analogous to the interaction between synMuv class A genes and *lin-35*).

In summary, the experiments described in this thesis accomplished three major goals. First, they helped to define the basic cell-cycle machinery used in *C. elegans*. Second, they demonstrated that cell-cycle regulation in *C. elegans* is accomplished using mechanisms that are conserved in mammals. Finally, building upon this foundation, forward genetic screens were used to identify novel genes involved in developmental control of cell division.

Chapter 1

Control of Cell Division in Multicellular Organisms

Mike Boxem

CONTENTS

INTRODUCTION

Research on cell division over the past decades has given us a detailed understanding of the machinery that controls passage through the cell division cycle. In most studies, cell division is examined in terms of how the divisions of individual cells are controlled. However, regulating cell division in a multicellular organism adds a whole new level of complexity. Growth, proliferation and differentiation of individual cells need to be coordinated with the development of the entire organism. The decision whether to divide or not is made largely in G_1 phase of the cell-cycle, and it is mainly during this phase that developmental cues appear to affect progression through the cell cycle. Many of the players that regulate progression through G_1 phase have been identified. These include the retinoblastoma protein family, the E2F transcription factors, G_1 cyclin dependent kinases and cyclin dependent kinase inhibitors. Much of what is known about the function of these proteins comes from studies of mammalian cells in tissue culture. More recently focus has shifted somewhat toward examining the roles of these key G_1 regulators in the development of multicellular organisms. In this introduction, I will first present an overview of the basic cell-cycle machinery and introduce the major players of G_1 regulation. In the other chapters of this thesis, we studied the functions of several G_1 regulators in *C. elegans*. These include *C. elegans* homologs of Cyclin D-CDK4/6, retinoblastoma family members, and E2F/DP transcription factors. In the second part of this chapter I will discuss the contributions that studies of these G_1 regulators in animal model systems have made toward our understanding of the process of G_1/S progression. Although homologs of G_1 regulators are present in a large variety of multicellular organisms, for the purpose of this introduction I will focus on studies performed in mice, *Drosophila melanogaster* and *Caenorhabditis elegans*.

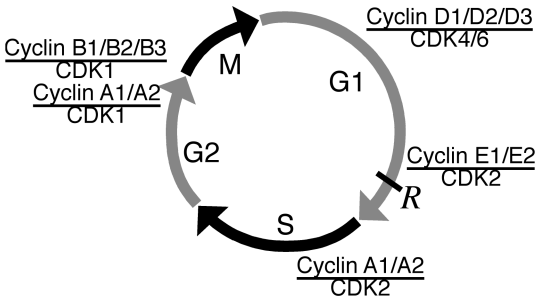

Fig. 1. Different Cyclin/CDK complexes regulate progression through the various phases of the cell cycle in higher eukaryotes. A eukaryotic cell cycle with the four major phases is shown. Cyclin D-dependent kinases are proposed to act early in G_1 phase in response to growth factor signaling. Cyclin E/CDK2 kinases are required for entry into S-phase. Cyclin A/CDK2 kinases are active during S-phase. Cyclins A and B in combination with CDK1 are required for entry into mitosis. R = restriction point.

THE EUKARYOTIC CELL CYCLE

Cell division in eukaryotic organisms generally consists of two major events (Nasmyth, 1996): the duplication of the genetic material during S phase and the segregation of the two copies into sister cells during mitosis. In a typical cell-division cycle, S-phase and mitosis are separated by two gap phases, called G_1 (before S-phase) and G_2 (between S- and M-phases). The time a cell spends in each phase varies between organisms and between cell types, and can depend on the extracellular environment. Variations on this general scheme do exist. For example, in the fruit fly *Drosophila melanogaster* early embryonic divisions lack gap phases (Edgar and Lehner, 1996). Many organisms also undergo specialized cell cycles where the DNA is replicated multiple times without cell division to form polyploid nuclei (Edgar and Orr-Weaver, 2001).

Regulation of cell-cycle progression by Cyclin Dependent Kinases

The transitions between the successive phases of the cell cycle are controlled by the activities of cyclin dependent kinases (CDKs) (Nigg, 1995). The founding members of the CDK family are the highly homologous 34kDa proteins encoded by the *cdc2* gene in the fission yeast *Schizosaccharomyces pombe* and the *CDC28* gene in the budding yeast *Schizosaccharomyces cerevisiae* (Beach *et al.*, 1982; Hartwell *et al.*, 1974; Nurse and Bissett, 1981). The first vertebrate CDK was identified as the catalytic subunit of the Maturation Promoting Factor complex (MPF), which promotes progression through M phase in egg extracts from the African clawed frog *Xenopus laevis*. In addition to the CDK subunit, active MPF complexes contain a regulatory subunit termed “cyclin”, as its abundance varies throughout the cell cycle. MPF activity fluctuates during the early cleavage stages of the *Xenopus* embryo, rising before cells enter mitosis and falling as they leave mitosis. This periodicity is controlled in part by the tightly controlled fluctuation in cyclin levels (reviewed in Murray and Kirschner, 1989).

In yeasts, a single CDK drives progression through the cell cycle in combination with different cyclin subunits. In mammalian cells, multiple CDK subunits have been identified in addition to a rather large family of Cyclins. Fig. 1 illustrates the different CDKs and cyclins identified in mammals and their proposed point of activity in the cell cycle (reviewed in Nigg, 1995). Surprisingly, very few critical CDK targets have been identified. This is probably because the effect of CDKs is achieved by phosphorylation of a multitude of targets, each of which on their own may not be rate limiting for cell-cycle progression. However, a few critical targets have been identified, including the retinoblastoma tumor suppressor product (pRb), which appears to be regulated by CDKs acting in G_1 phase (see below).

Regulation of CDK activity

Consistent with their important role in cell cycle regulation, the activity of Cyclin/CDK complexes is strictly controlled at several levels. The primary mechanism to control CDK activity is by regulating the levels of cyclins. Both protein synthesis and protein degradation play an important role in controlling the abundance of cyclins. Cyclin degradation is controlled by specific ubiquitin conjugating complexes that target cyclins for degradation by the 26S proteasome (Hershko, 1997; Hoyt, 1997). For example, the anaphase promoting complex (APC) targets B-type cyclins for destruction in mitosis, and Cyclin E is targeted for destruction by SCF (for Skp-1/Cullin/F-box) during S-phase (Peters, 1998; Winston *et al.*, 1999). A second level of control over Cyclin/CDK activity is accomplished by CDK phosphorylation and dephosphorylation. Phosphorylation of $p34^{CDC2}$ on threonine 14 and tyrosine 15 inhibits its kinase activity, and these inhibitory phosphorylations must be removed by the dual specificity phosphatase CDC25 before a cell can enter mitosis. In addition, phosphorylation on threonine 161, the third phosphorylation site of $p34^{CDC2}$, is required for kinase activity (see Nigg, 1995; Solomon, 1993 for reviews on CDK phosphorylation). Finally, Cyclin/CDK activity can be blocked by CDK inhibitors. Two classes of CDK inhibitors exist in mammalian cells (reviewed in Sherr and Roberts, 1999). Members of the INK4 family specifically inhibit the D-type cyclin activated kinases CDK4 and CDK6, while the Cip/Kip family of kinase inhibitors likely blocks CDK2 activity primarily. Together, these mechanisms ensure a tightly regulated peak of CDK activity at the appropriate time.

Developmental control of cell-cycle progression

During development, multicellular organisms need to integrate individual cell divisions with the developmental program of the whole organism. One of the means by which multicellular organisms can control cell divisions is through extracellular signals. A wide range of extracellular factors determines the rate of proliferation of cells. For example, mating pheromone can induce yeast cells to withdraw from the cell cycle and instead enter a sexual development program (Woollard and Nurse, 1995). In addition, yeast cells will stop cycling under conditions of limited nutrient availability. Similarly, mammalian cells in culture are dependent on growth factors in their medium to proliferate and often need to be anchored to a substrate (Pardee, 1989). A well known inhibitory signal for tissue culture cells is contact with neighboring cells (contact inhibition), which results in an arrest in G_1 phase.

The importance of G_1 phase for regulation of cell division was realized early on from studies on mammalian cells in culture by Arthur Pardee and Howard Temin (Pardee, 1974; Temin, 1971). They demonstrated that growth factor or nutrient withdrawal does not prevent progression through the cell cycle past a certain point in G_1 phase,

which was termed the restriction point. A similar transition point exists in yeast. At the onset of a new division cycle, both budding and fission yeast have a choice between entering a mitotic cell cycle or undergoing sexual development which entails a meiotic division. Past a certain point in G_1 phase, termed START, yeast cells are committed to the mitotic cycle and can only enter sexual development once they reach the next G_1 phase (reviewed in Woollard and Nurse, 1995). In addition, nutrient availability no longer affects cell-cycle progression once cells have passed START.

It is now generally accepted that many extracellular signals affect progression through the cell cycle during a window of opportunity in G_1 phase, and that cells past the restriction point are relatively unresponsive to extracellular stimuli. As is usually the case in biology, exceptions do exist. For example, many developmental signals in *Drosophila* affect entry into mitosis rather than S phase (Duronio, 1999).

Control of progression through G_1 phase by the Retinoblastoma family

To ensure that cells only enter a cell division cycle at the appropriate time, an elaborate system of checks and balances has evolved that regulates progression through G_1 into S phase. The importance of these control mechanisms is underscored by the fact that most, if not all, human tumor cells have defects in one or more of the pathways that control G_1 progression (Sherr, 1996). The product of the retinoblastoma gene, pRb, plays a pivotal role in the regulation of cell proliferation. Rb was originally identified as the gene mutated in familial retinoblastomas, a malignant tumor of the retina. Since somatic mutations that inactivate Rb have been found in a variety of different tumors, these and other studies have established that pRb acts as a tumor suppressor which normally restricts proliferation (reviewed in Cobrinik *et al.*, 1992).

pRb can exist in several phosphorylation states. The hypophosphorylated form of pRb acts as a negative regulator of cell cycle progression, as indicated by a number of different observations. First, viral oncoproteins produced by several DNA tumor viruses specifically bind to and inactivate hypophosphorylated pRb (Cobrinik *et al.*, 1992). Second, in cells synchronously released from G_1 arrest, pRb proteins become hyperphosphorylated around the time of passage through the restriction point, and remain hyperphosphorylated until sometime in late mitosis (reviewed in Weinberg, 1995). Finally, hypophosphorylated pRb was found to specifically bind to and control a number of cellular proteins, most notably the E2F transcription factors (Weinberg, 1995). These observations led to the now generally accepted conclusion that pRb phosphorylation is an essential step in progression from G_1 into S phase.

In mammals, three Rb family members exist: pRb, p107 and p130 (see Classon and Dyson, 2001 for a review on the similarities and differences between pRb, p107 and p130). All three proteins are structurally

very similar, especially in the domains bound by viral oncoproteins. In addition, all three family members are excellent substrates for phosphorylation by CDKs *in vitro*, and all three associate with members of the E2F family of transcription factors (see below). Despite these similarities, several differences do exist. First, the expression patterns are different for each family member, with pRb being expressed fairly constantly throughout G_1, p130 being expressed highly in G_0 and early G_1, and p107 levels rising at the end of G_1. Second, as described below, differences exist in the members of the E2F family bound by each pRb-related protein. Finally, p107 and p130 can bind to Cyclin A and Cyclin E-dependent kinase complexes. Most of the studies described in this chapter focus on the function of pRb, as this is the most extensively studied member of the pRb family, and the only one found to act as a tumor suppressor.

Phosphorylation of pRb by cyclin dependent kinases

In vitro, both Cyclin D-dependent kinases and Cyclin E/CDK2 are able to phosphorylate pRb (reviewed in Mittnacht, 1998). Cyclin E/CDK2 kinase activity peaks just before the onset of S-phase, and this activity is essential for entry into S-phase (Ekholm and Reed, 2000; Sherr, 1993). Transcription of Cyclin E during the G_1/S transition is controlled by E2F/DP transcription factors, which are negatively regulated by pRb (see below). As pRb itself is a target for Cyclin E/CDK2, this allows a feed-forward loop, with pRb inactivation increasing Cyclin E/CDK2 activity resulting in further inactivation of pRb.

In contrast to Cyclin E, the levels of Cyclin D remain fairly constant throughout the cell cycle. However, in response to growth factor withdrawal the levels of Cyclin D drop rapidly (see Sherr, 2000 for a review). In addition, growth factors posttranscriptionally regulate assembly of D-type cyclins with their catalytic CDK partners. D-type cyclins are, therefore, often referred to as growth factor sensors.

Cyclin D- and Cyclin E-dependent kinases phosphorylate pRb at distinct but overlapping subsets of sites, and phosphorylation of pRb appears to happen in a sequential fashion by first Cyclin D-CDK4/6 and then Cyclin E/CDK2 (Mittnacht, 1998). In one model of pRb inactivation, initial phosphorylation by Cyclin D-CDK4/6 inactivates pRb repressor complexes (see below), while subsequent phosphorylation by Cyclin E/CDK2 inhibits the ability of pRb to bind to and inactivate E2F/DP transcriptional activators (Harbour *et al.*, 1999). It should be noted however that the exact mechanisms of pRb inactivation by CDKs remain the subject of intense study and speculation.

The regulation of E2F transcription factors by pRb

The most widely studied effectors of pRb are the E2F transcription factors, and many of the effects of pRb on cell cycle progression are thought to be mediated by E2F (see Beijersbergen and Bernards, 1996

and; Dyson, 1998 for in depth reviews of E2F transcription factors). Although referred to as E2F, these transcription factors are actually heterodimers consisting of an E2F subunit and a DP subunit. In mammals, at least six E2F and two DP family members exist, and homologs of E2F and DP have been identified in many other organisms.

Hypophosphorylated pRb family members can stably bind E2F/DP complexes, and specific combinations are preferentially formed during particular phases of the cell cycle (see Table 1). At least two mechanisms have been described by which binding of pRb to E2F complexes blocks progression through G_1 phase. First, binding of pRb family members to transcriptionally active E2F/DP complexes likely prevents them from activating the transcription of S-phase genes. Second, E2F/DP can recruit pRb to the promoters of specific genes to form actively repressing complexes. Active transcriptional repression by pRb/E2F/DP most likely involves the recruitment of chromatin remodeling complexes to the promoter by pRb. At least three chromatin remodeling complexes have been implicated in this process. These are complexes with histone deacetylating activity, the Swi/Snf ATP-dependent chromatin remodeling complex and histone methylases (Harbour and Dean, 2000a; Harbour and Dean, 2000b; Nielsen *et al.*, 2001)
.

Table 1. Rb-family member/E2F complexes during the cell cycle

pRb family member	E2F family member	cell-cycle phase
pRb	E2F1,2,3,4	G_0, G_1, S
p107	E2F4	G_1, S, G_2
p130	E2F4,5	G_0, G_1

Overview of the most prevalent complexes between pRb and E2F family members formed in specific phases of the cell cycle (from Dyson, 1998).

Several additional mechanisms exist to regulate E2F activity. For example, E2F1 can be phosphorylated on Ser-332 and Ser-337 which blocks pRb from binding (Fagan *et al.*, 1994). Also, the transcription of certain E2F members may depend on other E2F proteins. For example, transcription of E2F-1 and E2F-2 appears to be inhibited by E2F4 and E2F5 complexes (reviewed in Dyson, 1998).

Taken together, the studies described above suggest a model for the regulation of G_1 progression in which a linear Cyclin D-CDK4/6 - pRb - E2F pathway takes center stage (summarized in Fig. 2). This model leaves several important questions unanswered. Chief among these is the *in vivo* importance of the various proposed G_1 regulators. Another very important question is the issue of functional redundancy between multiple family members. Mammalian cells have three Rb family members, six E2Fs, two DPs, three D-type cyclins and two kinase partners for cyclin D. Do all these family members simply provide redundant functions, or do they perform their own specialized functions? With the more recent development of mice deficient in one or more G_1 regulators, these and other questions are starting to be addressed. Studies of these mechanisms in the model organisms *Drosophila melanogaster* and *Caenorhabditis elegans* also provide a valuable contribution. In particular, studies of G_1 regulators in these organisms are aided by the fact that families of G_1 regulators contain far fewer members. This reduces or eliminates potential problems in interpreting results arising from functional redundancy between family members. In addition, powerful genetic methods are available to analyze interactions between the various G_1 regulators, and forward genetic screens can be used to identify novel components that act in G_1 control *in vivo*.

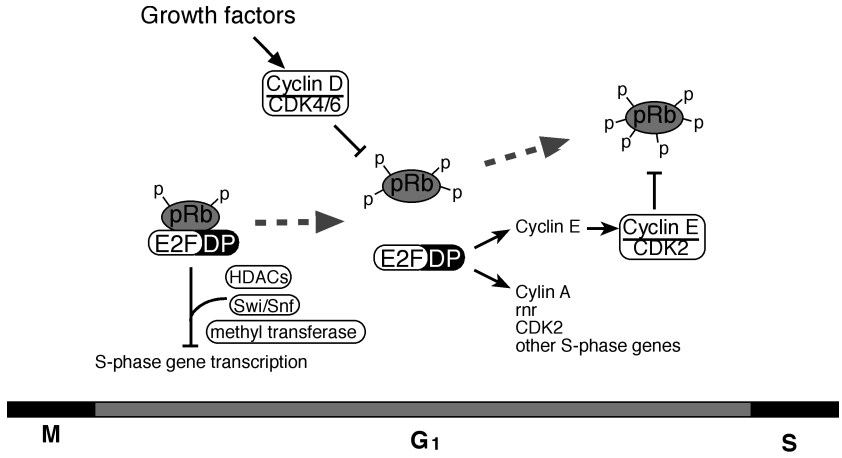

Fig. 2. Model for the control of G_1 progression by D-type cyclins, pRb and E2Fs. In growth arrested cells, pRb exists mainly in the hypophosphorylated form and binds to E2F/DP complexes. This has two effects. On the one hand, E2F/DP transcription factors are prevented from activating genes required for S-phase entry. On the other hand, pRb/E2F/DP complexes can actively block transcription, which may involve recruitment by pRb of additional factors such as Histone Deacetylases and chromatin modifying complexes like Swi/Snf and methylases. Early in G_1, D-type cyclin dependent kinases are formed, likely in response to growth factor signaling. These initiate the phosphorylation and inactivation of pRb. This results in release of E2F/DP transcription factors which can now activate genes required for S-phase entry. One E2F target gene is Cyclin E, which associates with CDK2 and helps to further inactivate pRb.

THE CYCLIN D-CDK4/6 – PRB – E2F PATHWAY IN VIVO

In the rest of this chapter, I will discuss the functions of the pRb pathway in multicellular organisms. I will focus on Cyclin D-CDK4/6, the pRb family and E2F/DP transcription factors, as the studies described in this thesis deal largely with the functions of these cell-cycle regulators in *C. elegans*. It is important to realize however that many components not discussed in this Chapter also affect the pRb pathway. For example, E2F transcription factors are just one of many potential pRb binding partners, although they are the most widely studied. Other candidate Rb partners include the chromatin remodelling complexes mentioned above, and the Id2 protein. Id2 is a dominant-negative antagonist of basic helix-loop-helix transcription factors. Importantly, loss of Id2 in mice rescues the embryonic lethality associated with pRb knockout mice (Lasorella *et al.*, 2000). See (Morris and Dyson, 2001) for a comprehensive review of potential pRb partners.

Another important regulator of G_1 progression is the INK4 family. Members of the INK4 family inhibit CDK4/6 kinases and have been implicated as tumor suppressors. However, no clear INK4 family members have been identified in *C. elegans*, and it would therefore be beyond the scope of this thesis to discuss this family in detail.

The Rb family

The functions of pRb in mammalian development

The first Rb family member whose function was examined *in vivo* was pRb itself. Rb deficient mice complete a substantial part of embryogenesis, but ultimately die in mid-gestation with severe defects in erythropoiesis as well as neuronal and lens development (Clarke *et al.*, 1992; Jacks *et al.*, 1992; Lee *et al.*, 1992). Close examination of the defects in $pRb^{-/-}$ mice indicates that pRb plays diverse roles during development *in vivo*. Embryos deficient for pRb show severe defects in the differentiation of multiple tissues. For example, pRb mutant mice have smaller fetal livers and a dramatic increase in the number of immature nucleated erythrocytes (Jacks *et al.*, 1992; Lee *et al.*, 1992). This appears to be due to a failure of erythroid precursor cells to differentiate into mature, enucleated red blood cells (Jacks *et al.*, 1992; Robanus Maandag *et al.*, 1994). pRb deficiency also affects the differentiation of many nerve cells, especially in the sensory ganglia. These cells do not mature properly, and fail to express many neuronal markers, such as βII tubulin, Trk A, Trk B, and the low-affinity neurotrophin receptor $p57^{LNGFR}$ (Lee *et al.*, 1994). Differentiation of lens fiber cells is also abnormal in $Rb^{-/-}$ mice, as expression of many late lens differentiation markers, such as β-crystallin and filensin, is impaired (Liu and Zacksenhaus, 2000; Morgenbesser *et al.*, 1994). Finally, differentiation of cultured myotubes from $pRb^{-/-}$ mice was impaired as evidenced by a reduction in the expression level of late

muscle specific genes (Zacksenhaus *et al.*, 1996). Thus, pRb appears to be involved in the process of cell differentiation. Early differentiation markers are often still expressed, but late markers are not. In general the tissues that are most severely affected by loss of pRb are those that normally have high pRb expression levels.

In addition to being involved in differentiation, pRb appears to be required for cells to exit the cell cycle and to keep cells in a quiescent state. Ectopic S phases are observed in multiple tissues of $pRb^{-/-}$ mice. These include the central and peripheral nervous systems (CNS and PNS), and lens fiber cells in the developing lens (Clarke *et al.*, 1992; Jacks *et al.*, 1992; Lee *et al.*, 1994; Lee *et al.*, 1992; Morgenbesser *et al.*, 1994). In skeletal muscle cells, pRb appears to be required to keep myotubes in a quiescent state. MyoD can still induce cultured skeletal muscle cells from $Rb^{-/-}$ mice and $Rb^{-/-}$ MEFs to differentiate into mature myotubes, but these myotubes can re-enter the cell cycle when stimulated with growth factors, in contrast to wild type myotubes (Novitch *et al.*, 1996; Schneider *et al.*, 1994).

Finally, the presence of pRb appears to protect cells from undergoing apoptosis, as evidenced by the finding that many tissues in pRb deficient mice demonstrate high levels of apoptosis. For example, both the CNS and PNS show exceptionally high levels of apoptosis (Clarke *et al.*, 1992; Jacks *et al.*, 1992; Lee *et al.*, 1994; Lee *et al.*, 1992). In addition, increased levels of apoptosis are found in both lens fiber cells and skeletal muscle cells (Morgenbesser *et al.*, 1994; Jiang *et al.*, 2000). The p53 gene product is a potent mediator of apoptosis, and cell death in most of the tissues of $pRb^{-/-}$ mice described above indeed depends on the activity of p53 (Macleod *et al.*, 1996; Morgenbesser *et al.*, 1994; Tsai *et al.*, 1998). However, apoptosis in the PNS and skeletal muscle appear to be independent of p53 activity (Jiang *et al.*, 2000; Macleod *et al.*, 1996).

Together, these results provide strong support that pRb not only regulates G1 progression *in vivo*, but also is involved in processes such as cell differentiation, cell-cycle exit and protection against apoptosis.

pRb deficiency and tumor formation

As would be expected from the loss of an important tumor suppressor, $pRb^{+/-}$ mice are indeed prone to tumor development. The two most prevalent types of tumor that develop in $pRb^{+/-}$ mice are thyroid C-cell adenomas and pituitary adenocarcinoma's of the intermediate lobe (Hu *et al.*, 1994; Jacks *et al.*, 1992; Robanus Maandag *et al.*, 1994; Williams *et al.*, 1994a; Williams *et al.*, 1994b; Yamasaki *et al.*, 1998). Surprisingly, $pRb^{+/-}$ mice do not develop retinoblastomas (Clarke *et al.*, 1992; Jacks *et al.*, 1992; Lee *et al.*, 1992). However, inactivation of the Rb family by expression of HPV-16 E7 in mice deficient for p53 does result in the development of retinoblastomas, indicating that the activity of p53 may prevent the formation of retinoblastomas in $pRb^{+/-}$ mice (Howes *et al.*,

1994). A detailed description of tumor predisposition in Rb mutant mice can be found in (Vooijs and Berns, 1999).

The functions of p107 and p130 in mammalian development

The effects of inactivation of p107 or p130 are generally much less severe than those that result from pRb inactivation. However, the severity is dramatically affected by the genetic background; while inactivation of p107 or p130 in a C57BL/6J background has minimal effects, much more severe defects are observed in a Balb/c background. In a C57BL/6J background, p107 deficient mice show a minimal thickening of the bones of the forelimbs (Cobrinik *et al.*, 1996; Lee *et al.*, 1996). By contrast, in a Balb/c background the loss of p107 results in a reduced rate of postnatal growth combined with a compromised immune system and a myoproliferative disorder, which results in extensive extramedullary haematopoiesis in liver and spleen (LeCouter *et al.*, 1998a). Similarly, in a C57BL/6J background the loss of p130 causes no overt phenotype, while in a Balb/c background, loss of p130 causes embryonic death between E11 and E13 with defects in proliferation of skeletal and cardiac muscle and neurons in the neural tube and dorsal root ganglia (LeCouter *et al.*, 1998b). These results suggest the presence of a second site modifier in the Balb/c background that is responsible for the more severe phenotype. Interestingly, Balb/c mice have a non-functional $p16^{INK4a}$ gene (Zhang *et al.*, 1998). As p107 and p130, as well as $p16^{INK4a}$, can inhibit G_1 progression, the loss of p16 and p107 or p130 might produce an additive effect.

The examination of p107/p130 double knockout mice revealed a significant functional redundancy between p107 and p130. First, such mice die just after birth, due to an inability to breath. In addition, the double knockout mice have far more severe defects in bone formation than either single knockout, as evidenced by their dramatically shortened limbs (Cobrinik *et al.*, 1996). The defect in bone formation is found in all bones formed by endochondral ossification. Endochondral ossification requires chondrocytes to exit the cell cycle and undergo hypotrophy, which is normally followed by replacement of these cells by invading osteocytes. In $p107^{-/-}/p130^{-/-}$ mice chondrocyte density is increased 2-fold in areas where chondrocytes should normally exit the cell cycle, and many of these cells are still actively entering S-phase. As these defects are not observed in the single knockouts, p107 and p130 thus appear to act redundantly in restricting chondrocyte proliferation (Cobrinik *et al.*, 1996). These results demonstrate that p107 and p130 have largely overlapping functions that are distinct from those of pRb.

Functional redundancy between all three Rb family members

The observations described in the previous section clearly indicate that although individual Rb family members appear to play distinct roles during development, significant overlap exist between the functions of

at least p107 and p130. Possible overlap between p107 and pRb is indicated by the finding that mice that lack both Rb and p107 die earlier and show high levels of apoptosis in the CNS and liver at an earlier time point than $Rb^{-/-}$ mice (Lee *et al.*, 1996). Recently, the effects of knocking out all three Rb-family members have been examined in mouse embryonic fibroblasts (MEFs) (Dannenberg *et al.*, 2000; Sage *et al.*, 2000). Complete inactivation of the Rb family leads to a dramatic decrease in population doubling times of MEFs. The percentage of cells in G_1 phase is decreased compared to wild-type MEFs, concomitant with an increased S-phase population. In addition, the triple knockout MEFs are immortalized and fail to arrest in G_1-phase in response to inhibitory signals like DNA-damage, confluence, low serum or senescence. Thus, these cells have further lost cell-cycle control compared to cells lacking one or two Rb-family members. Loss of Rb and either p107 or p130, or double inactivation of p107 and p130 leads to a shortening of G_1 phase that is compensated for by an extension of S phase, leaving the total doubling time unaffected (Classon *et al.*, 2000; Herrera *et al.*, 1996; Hurford *et al.*, 1997). These cells are also still responsive to serum withdrawal. From these experiments it becomes clear that pRb, p107 and p130 perform at least partially overlapping functions.

Cell autonomous versus non cell-autonomous functions of pRb

Given the embryonic death of $Rb^{-/-}$ mice, it was surprising to find that chimeric mice with high contributions of $Rb^{-/-}$ ES cells have only minor defects (Robanus Maandag *et al.*, 1994; Williams *et al.*, 1994b). In chimeric mice, both Rb^{+} and $Rb^{-/-}$ cells contribute to erythropoiesis. As these mice show normal numbers of enucleated red blood cells, the erythropoiesis defect found in $Rb^{-/-}$ mice does not appear to be a cell-autonomous defect of Rb loss (Robanus Maandag *et al.*, 1994; Williams *et al.*, 1994b).

Recently, the apoptosis in the CNS of chimeric pRb mice was examined in more detail (Lipinski *et al.*, 2001). Compared to $Rb^{-/-}$ embryos, the number of apoptotic cells in the CNS at E13.5 was significantly lower in chimeric embryos. This suppression of apoptosis does not appear to be due to complete rescue of the $Rb^{-/-}$ defects, as the level of ectopic cell cycle entry of $Rb^{-/-}$ cells in chimeric mice is comparable to that found in germline $Rb^{-/-}$ embryos. Thus, like the erythropoiesis differentiation defect, apoptosis in $Rb^{-/-}$ cells is rescued by neighboring wild-type cells. This rescue appears to occur downstream of activation of p53, as p53 protein levels and DNA binding activity are still elevated in $Rb^{-/-}$ chimeric mice. As ectopic S-phase entry is not suppressed in these chimeras, these data demonstrate that in mice, Rb likely has a cell-autonomous function in cell-cycle entry and a non cell-autonomous function in differentiation and prevention of apoptosis. In addition, the finding that apoptosis can be suppressed independently from the

ectopic S-phase entry may indicate that apoptosis is not a necessary consequence of ectopic S-phase entry.

The function of the fly Rb family member RBF1

The *Drosophila* genome contains two genes homologous to Rb family members, of which only one, *rbf1*, has been studied to date. *rbf1* mutant flies die at an early larval stage (Du, 2000; Du and Dyson, 1999). By using such *rbf1* mutants in combination with by heat shock induced expression of wild-type RBF1, Du *et al.* demonstrated an absolute requirement for RBF1 during early larval development, and a requirement for RBF1 in bristle and eye development in later stages of development (Du, 2000). The *Drosophila rbf1* mutation primarily affects the G_1 arrest of epidermal cells following mitosis 16. In wild-type flies, early development consists of a series of cell cycles that lack detectable G_1 phases. Following embryonic division 16, epidermal cells enter their first G_1 phase and permanently arrest cell divisions. In *rbf1* mutants, the epidermal cells enter G_1 following mitosis 16, but they are unable to remain in G_1 as evidenced by incorporation of BrdU (Du and Dyson, 1999). These cells do no appear to re-enter M-phase. In agreement with a role for RBF1 as a negative regulator of G_1 progression, ectopic expression of RBF1 in the developing eye or wing caused a decrease in cell divisions (Du *et al.*, 1996a; Neufeld *et al.*, 1998). Thus, similar to pRb in mice, RBF1 appears to be required to keep cells in a quiescent state.

Similar to pRb deficient mice, extensive apoptosis is observed in the epidermis of *rbf1* mutant embryos. However, as the cells that enter S-phase are not the same as those that undergo apoptosis, apoptosis is not a direct consequence of ectopic S-phase.

The functions of lin-35 *Rb in* C. elegans

The *C. elegans* genome contains a single Rb family member, *lin-35*, which was originally identified as a member of the synthetic multivulva (synMuv) gene family (Lu and Horvitz, 1998). The synMuv genes counteract a Ras-mediated signal that induces vulval cell fates. Loss of synMuv activity causes too many cells to adopt the primary vulval cell fate, leading to the formation of multiple pseudo-vulvae along the ventral side of the animal (Ferguson and Horvitz, 1985; Ferguson *et al.*, 1987). Genetically the synMuv genes fall into two classes, A and B, and the appearance of the multivulva phenotype requires the inactivation of both a class A and a class B gene (Ferguson and Horvitz, 1989). *lin-35* Rb acts as a class B synMuv gene. Surprisingly, *lin-35* Rb mutants appear to develop normally and become fertile adults, indicating that loss of *lin-35* Rb has no effect on cell divisions. However, we recently showed that *lin-35* Rb does act as a negative regulator of G_1 progression by examining the effect of *lin-35* Rb loss of function in combination with other cell-cycle mutants (see Chapter 3). Loss of *cyd-1* Cyclin D or *cdk-4* CDK4/6 results in a block of cell division in G_1 phase. Inactivation of *lin-35* Rb in a *cyd-1* or

cdk-4 mutant background restores several rounds of cell division, endoreduplication in intestinal cells and expression of the S-phase marker *rnr::GFP* (Boxem and van den Heuvel, 2001). Further evidence that *lin-35* Rb acts to negatively regulate G_1 progression comes from the observation that *lin-35* Rb acts in a parallel pathway to the Cip/Kip family members *cki-1* and *cki-2*. Loss of *cki-1* and *cki-2* results in additional divisions of several cell types including the intestinal nuclei. While loss of *lin-35* Rb by itself does not cause additional divisions, double inactivation of the Rb and Cip/Kip families results in a synergistic increase in postembryonic divisions. Therefore, *lin-35* Rb and *cki-1* + *cki-2* Cip/Kip likely form two parallel pathways that negatively regulate proliferation (Boxem and van den Heuvel, 2001). These result demonstrate that *lin-35* Rb acts as a negative regulator of G_1 progression.

These studies on the function of the pRb family in animal models illustrate that Rb related genes may play diverse roles during development. In mice, pRb family members appear to function in differentiation, during exit from the cell-cycle and to protect against apoptosis. Similarly, in flies, RBF1 appears to be required to keep cells in a quiescent state and to prevent apoptosis. Finally, in *C. elegans*, *lin-35* Rb functions as a negative regulator of G_1 progression but also acts as a regulator of the vulval cell fate.

E2F transcription factors

Functional antagonism and redundancy between mammalian E2F family members

In mammals, six E2F genes and two DP genes have been found to date. Several observations indicate that E2F1, E2F2 and E2F3 act predominantly as transcriptional activators, while E2F4 and E2F5 may normally inhibit transcription (reviewed in Dyson, 1998). First, although overexpression of all E2F family members can activate transcription, E2F4 and E2F5 do so much less efficiently. In addition, only E2F1, E2F2 and E2F3 can drive quiescent cells into S-phase when overexpressed. Finally, while E2F1, E2F2 and E2F3 complexes localize to the nucleus, E2F4 and E2F5 lack a nuclear localization signal and depend on Rb family members for translocation to the nucleus. Thus, when pRb family members become phosphorylated, E2F4 and E2F5 actually become cytoplasmic and are presumably inactive. The function of E2F6, which lacks a transactivation and Rb binding domain is largely unknown. Two important questions about the functions of mammalian E2Fs are to what extent the functions of the E2F family members overlap, and which mode of action of E2F complexes is most important for the function of E2F *in vivo*.

The function of E2F1 was the first to be studied in a knockout mouse (Field *et al.*, 1996). Surprisingly, E2F1 deficient mice show only minor developmental abnormalities. $E2F1^{-/-}$ mice develop testicular atrophy,

and exocrine glands with enlarged nuclei that are often binucleate (Field *et al.*, 1996; Yamasaki *et al.*, 1996). In addition, the thymus of E2F1$^{-/-}$ mice is enlarged due to a decrease in apoptosis of immature thymocytes (Field *et al.*, 1996). In agreement with these mild defects, E2F1$^{-/-}$ MEFs do not have defects in cell-cycle progression or proliferation rates, and cells derived from lymph nodes proliferate normally in response to mitogen stimulation (Field *et al.*, 1996).

E2F3 knockout mice display more severe defects. Many E2F3$^{-/-}$ mice die before birth, indicating an important role for E2F3 in embryonic development (Humbert *et al.*, 2000b). In contrast to E2F1$^{-/-}$ MEFs, MEFs deficient for E2F3 proliferate slower with a delay in initiation of S phase and a reduced rate of DNA replication (Humbert *et al.*, 2000b). In addition, while in E2F1$^{-/-}$ MEFs only cyclin E expression is reduced, in E2F3$^{-/-}$ MEFs many E2F responsive genes show delayed expression and reduced levels. This indicates that E2F3 may be responsible for the transcription of many E2F target genes *in vivo*. When cells are released from a G_1 arrest, both E2F1 and E2F3 are downregulated after S-phase, but only E2F3 is upregulated in the next G_1 phase (Leone *et al.*, 1998). In addition, of the E2F target genes that are upregulated during entry into S from G_0, only a subset is cell-cycle regulated during the second division cycle. These data suggest a model where E2F1 functions during the exit from G_0 into G_1 phase, while E2F3 is required for entry into S-phase during normal proliferation.

Despite these differences in function, considerable overlap does exist between E2Fs 1-3. Overlap between E2F3 and either E2F1 or E2F2 is indicated by the much more severe phenotype of E2F1/E2F3 and E2F2/E2F3 double knockout mice, which both are embryonic lethal at day 9.5 of embryogenesis (Wu *et al.*, 2001). Recently, a knockout mouse was described that lacks E2F1,2 and in which E2F3 can be conditionally inactivated (Wu *et al.*, 2001). Triple E2F knockout MEFs derived from these mice were found to completely lack S-phase entry and cell proliferation. This result is the first demonstration that the function of mammalian E2F transcription factors is essential for progression into S-phase.

Mice lacking one of the second class of E2Fs, consisting of E2F4 and E2F5, show a very different phenotype than E2F1-3 knockout mice. E2F4 deficient mice have two limited defects. The first is a defect in differentiation of the haematopoietic cell lineage. Red blood cells show various abnormalities such as variation in size, increase in cell volume and Howell-Jolly bodies (nuclear remnants of improper enucleation) (Humbert *et al.*, 2000a; Rempel *et al.*, 2000). In addition, the mice have a craniofacial defect that results in accumulation of proteinaceous secretions in the nasal cavities, increasing the susceptibility to bacterial infection. These mice die 3-4 days after birth from a bacterial infection of the nasal passages (Humbert *et al.*, 2000a). MEFs lacking functional

E2F4 do not show defects in proliferation, cell cycle arrest in response to serum withdrawal or cell cycle entry after serum addition (Humbert *et al.*, 2000a).

E2F5 knockout mice develop hydrocephalus (abnormal buildup of cerebrospinal fluid in the ventricles of the brain) within the first few days of life (Lindeman *et al.*, 1998). The cause of the hydrocephalus is not completely known, but likely results from excess production of cerebrospinal fluid in the choroid plexus, a tissue in which E2F5 is highly expressed. E2F5 deficient MEFs also do not show altered cell-cycle kinetics compared to wild-type cells, and still respond to contact inhibition.

Mice deficient for both E2F4 and E2F5 die before birth, indicating an overlap in function between these two E2Fs (Gaubatz *et al.*, 2000). Strikingly, $E2F4^{-/-}/E2F5^{-/-}$ MEFs fail to arrest in G_1 phase in response to expression of the CDK4/6 inhibitor $p16^{INK4a}$, even though all other aspects of cell cycle regulation examined are normal (Gaubatz *et al.*, 2000). $p16^{INK4a}$ activity may arrest cells in G_1 phase by two mechanisms. First, it inhibits cyclin D dependent kinases, leading to an accumulation of hypophosphorylated Rb-family members. Second, binding of $p16^{INK4a}$ displaces $p21^{Cip/Kip}$ from cyclin D-CDK4/6, allowing it to bind to and inactivate Cyclin E/CDK2 complexes. $E2F4^{-/-}/E2F5^{-/-}$ MEFs still arrest in response to expression of $p21^{CIP}$, indicating that E2F4 and E2F5 are specifically required for the pocket protein mediated G_1 arrest in response to $p16^{INK4a}$. In this function they act redundantly since ectopic expression of either E2F4 or E2F5 restores the arrest in response to $p16^{INK4a}$.

The results obtained in mammalian cells thus demonstrate the existence of two distinct functions for E2Fs. While E2Fs 1-3 are positive regulators of G_1 progression, and their function is essential for cell division, E2F4 and E2F5 restrict G_1 progression under certain conditions.

The role of E2F in Rb induced cell-cycle entry and apoptosis

The E2F family of transcription factors is the most extensively studied target of pRb family members. However, over the years, a large number of proteins that can interact with pRb has been identified (Morris and Dyson, 2001). It is important therefore to determine to what extent the functions of pRb are affected through E2F family members *in vivo*. Examining the effect of E2F loss of function on the Rb mutant phenotypes is a powerful approach to determine the importance of E2F as an Rb target. In mice, the effects of loss of E2F1 and E2F3 on the $Rb^{-/-}$ phenotype have been examined. Loss of E2F1 or E2F3 extends the lifespan of Rb deficient embryos from mid-gestation (E13.5) to E17 or E18.5, respectively. E2F1 and E2F3 appear to be involved in both the ectopic S-phases and increase in apoptosis in Rb deficient mice. Loss of E2F1 or E2F3 in $Rb^{-/-}$ embryos suppresses ectopic S-phase entry and apoptosis in the lens and the CNS (Tsai *et al.*, 1998; Ziebold *et al.*, 2001). This is in

contrast to mammalian cells in tissue culture, where only expression of E2F1 was able to promote apoptosis (DeGregori *et al.*, 1997). These observations clearly established E2Fs as important pRb targets, and also indicate that specific E2Fs may perform different functions.

Apoptosis in the CNS and lens are p53 dependent. One mechanism by which E2F might promote apoptosis is through transcription of $p19^{ARF}$, which results in stabilization of p53 (reviewed in Phillips and Vousden, 2001). Consistent with this E2F function, loss of E2F1 or E2F3 do not rescue ectopic S-phase entry in skeletal muscle, which is p53 independent (Jiang *et al.*, 2000; Tsai *et al.*, 1998; Ziebold *et al.*, 2001). However, loss of E2F3 does rescue apoptosis in the PNS, which is also p53 independent, providing *in vivo* support for the idea that E2Fs can induce apoptosis through multiple mechanisms (Phillips and Vousden, 2001; Ziebold *et al.*, 2001).

Redundancy between Rb and E2F family members plays a much smaller role in *Drosophila* than in mammals. To date only two Rb-family members, two E2F-family members and one DP homolog are known. Several results indicate that dE2F1 is an important target for RBF1. As mentioned earlier, *Drosophila rbf1* mutants die at an early larval stage. This *rbf1* mutant phenotype can be rescued significantly by reducing the *de2f1* dosage, suggesting that dE2F1 mediates RBF1 functions during larval development. Similarly, reducing the levels of dE2F1 can rescue the bristle and eye defects that arise in *rbf1* mutants in which the early larval death is suppressed by briefly expressing RBF1 from a heat shock construct during early larval stages (Du, 2000). Finally, *rbf1* mutants can even develop into adult flies when the only source of dE2F1 is a mutant dE2F1 that lacks the Rb binding and transactivation domains (Du, 2000). Thus, the *rbf1* mutant phenotype appears to be due in large part to an increase in dE2F1 activity, and RBF1/dE2F1 appear to act together *in vivo*.

The function of *C. elegans efl-1* E2F bears striking resemblance to that of *lin-35* Rb (see Chapter 4 and below). Both *efl-1* and *lin-35* act as negative regulators of G_1 progression downstream of or in parallel to *cyd-1*. In addition, both *lin-35* and *efl-1* act as synMuv class B genes in vulval cell-fate specification. Given the resemblance between *efl-1* E2F and *lin-35* Rb loss-of-function phenotypes it is likely that much of the activity of LIN-35 RB is exerted in an inhibitory complex with EFL-1.

Functional antagonism between the two Drosophila *E2F homologs*

To date, two E2F homologs have been identified in *Drosophila*. dE2F1 has similar biochemical properties as mammalian E2Fs 1-3. dE2F1 can bind to E2F recognition sites in a complex with dDP, and expression of dE2F1 plus dDP stimulates transcription of E2F target promoters in *Drosophila* Schneider cells in tissue culture (Dynlacht *et al.*, 1994; Ohtani and Nevins, 1994). *Drosophila* dE2F1 appears to be required for progression through G_1 phase. Overexpression of dE2F1/dDP *in vivo* drives

normally quiescent cells into S-phase and stimulates apoptosis (Asano *et al.*, 1996; Du *et al.*, 1996b). In *de2f1* mutant flies, expression of many E2F target genes is severely reduced, and DNA replication is nearly absent after embryonic cycle 16 (Duronio *et al.*, 1998; Royzman *et al.*, 1997). Larvae hatch but grow very slowly and larval development is not completed. Early embryonic cell cycles are unaffected, presumably due to the maternal wild-type dE2F1 contribution (Duronio *et al.*, 1995). Finally, cells in *de2f1* mutant clones in eye imaginal disks do not enter S-phase (Brook *et al.*, 1996). Based upon these data, *de2f1* is an essential gene for G_1 progression.

The second E2F homolog in flies, dE2F2, can also cooperate with dDP in binding to E2F consensus sites *in vitro*. In contrast to dE2F1/dDP however, dE2F2/DP can repress transcription from the *PCNA* promoter, a known E2F target gene (Frolov *et al.*, 2001; Sawado *et al.*, 1998). Repression by dE2F2/dDP is not very strong and can be overcome by expression of low levels of dE2F1 (Frolov *et al.*, 2001). Overexpression studies also indicate that *de2f1* and *de2f2* perform opposing functions. Overexpression of dE2F2 in the developing eye results in a rough eye phenotype, which can be rescued by overexpression of dE2F1 and enhanced by the loss of one *de2f1* allele. The strongest argument for an opposing function of dE2F1 and dE2F2 comes from the analysis of *de2f1;de2f2* double mutant flies. As mentioned above, *de2f1* mutant embryos lack expression of E2F target genes, show almost no DNA synthesis and fail to complete larval development. Strikingly, *de2f1*$^{-}$*;de2f2*$^{-}$ larvae develop normally until they reach the pupal stage, where they finally die as mid to late pupae (Frolov *et al.*, 2001). Furthermore, in contrast to dE2F1 mutant eye imaginal disks, eye disks that lack both dE2F1 and dE2F2 have normal patterns of DNA replication and express cyclin A and a mitotic marker: phosphorylated histone H3. Although expression levels or patterns are frequently changed, E2F target genes are still expressed in doubly mutant eye imaginal disks, which may explain why cell proliferation can take place in the absence of any E2F activity. These results clearly demonstrate that dE2F1 and dE2F2 have opposing roles during development.

E2F functions in C. elegans

The *C. elegans* genome contains two E2F homologs, *efl-1* and *efl-2*, and one DP homolog, *dpl-1*. To date, no function has been demonstrated for *efl-2*. *efl-1* and *dpl-1* mutants were independently identified by two groups. Page *et al.* identified *efl-1* and *dpl-1* as genes required for the asymmetric localization of early embryonic determinants (Page *et al.*, 2001). Ceol *et al.* identified *dpl-1* and *efl-1* as genes that act in the synthetic Multivulva (synMuv) pathway for vulval cell fate specification (Ceol and Horvitz, 2001). In both cases, *efl-1* and *dpl-1* antagonize a Ras/MAPK signal. The strongest indication that these genes act in a linear pathway is that loss of *efl-1* or *dpl-1* causes increased MAPK activity in

the developing oocyte. The targets of MAPK activity in the embryo remain unknown.

Surprisingly, a role in cell-cycle regulation for *efl-1* and *dpl-1* was not described. The only effect on G_1 progression described for *efl-1* and *dpl-1* was a reduced number of P-neuroblast divisions in *dpl-1* mutant animals (Ceol and Horvitz, 2001). However, we recently showed that similar to *lin-35* Rb, loss of *efl-1* overcomes the G_1 arrest found in *cyd-1* mutant animals (see Chapter 4). In addition, the role of *efl-1* as a negative regulator of cell-cycle entry also becomes apparent when inactivation of *efl-1* is combined with inactivation of *cki-1* Cip/Kip. Inactivation of *efl-1* E2F and *cki-1* Cip/Kip results in a synergistic increase in ectopic cell divisions. These data indicate that EFL-1 acts primarily as a negative regulator of G_1 progression. In agreement with this conclusion, the closest mammalian homologs of EFL-1 are E2F4 and E2F5. Closer examination of *dpl-1* DP reveals a dual role as a positive and a negative regulator of G_1 progression. Inactivation of *dpl-1* overcomes the G_1 arrest of *cyd-1* mutant animals. However, inactivation of *dpl-1* in wild-type animals results in fewer P-neuroblast and intestinal divisions. Furthermore, loss of *dpl-1* suppresses expression of GFP under control of the ribonucleotide reductase major subunit promoter, a known E2F target gene. These data suggest that a second E2F forms an activating complex with DPL-1, similar to the two E2F complexes described in *Drosophila*.

The studies on the function of E2F described suggest that in higher eukaryotes two species of E2F/DP complexes exist that perform opposing functions. One complex stimulates progression through G_1 into S phase while the other type inhibits this transition.

The role of E2F in tumorigenesis

The function of E2F1-3 as promoters of cell-cycle progression indicated that these genes might act as oncogenes *in vivo*. Many tumor cells have sustained mutations that inactivate pRb and thus result in activation of E2F1-3. Surprisingly, E2F1 knockout mice were found to be prone to tumor development (Yamasaki *et al.*, 1996). Up to 34% of the mice were found to develop tumors, mostly sarcomas of the reproductive tract, within the first 18 months of life (Yamasaki *et al.*, 1996). This indicated that *in vivo* E2F1 acts as a tumor suppressor gene. One mechanism by which wild type E2F1 might prevent tumor formation is by inducing apoptosis. As described above, loss of E2F1 suppresses the high levels of apoptosis found in Rb mutant mice.

Consistent with a role as a tumor suppressor, inactivation of E2F1 in heterozygous Rb mutant mice, results in a general increase of tumor incidence. However, a tissue specific component to the role of E2F in tumorigenesis may exist, as the number of C-cell hyperplasias/adenomas was actually reduced (Yamasaki *et al.*, 1998). Such tissue specificity might be the result of a balance between two functions of E2F1: one

promoting progression through G_1 phase and one inducing apoptosis. This conclusion is consistent with results obtained in a mouse model for brain tumors induced by expression of a truncated SV40 large T oncogene. In this system, loss of p53 leads to aggressive tumor growth due to a large reduction in apoptosis (Symonds *et al.*, 1994). Loss of E2F1 causes a similar reduction in apoptosis, but does not lead to aggressive tumor growth, most likely because cell proliferation is reduced simultaneously (Pan *et al.*, 1998).

A role for E2F in other phases of the cell cycle

Although most studies on E2F function have focused on a role for E2F specifically in G_1 phase, several observations indicate that the requirement for E2F activity is not limited to G_1 phase. E2F function is required during S-phase for the accumulation of Cyclin B1, a G_2/M cyclin which triggers mitosis (Lukas *et al.*, 1999). Recently, micro-array analysis was performed to identify genes whose expression is regulated by E2Fs 1-3 (Ishida *et al.*, 2001; Muller *et al.*, 2001). These studies revealed that in addition to G_1 cell-cycle regulators, E2F appeared to regulate the expression of genes acting in G_2/M, apoptosis and differentiation. Perhaps the most conclusive support for a role of E2F1-3 outside of G_1 phase comes from the triple knockout MEFs. Loss of E2F1, E2F2 and E2F3 arrested cell-cycle progression in all phases of the cell-cycle, rather than causing a specific G_1 arrest (Wu *et al.*, 2001).

Compelling evidence also exists for a role for *Drosophila* E2F outside of G_1 phase. Overexpression of dE2F1 results in shorter doubling times and shorter gap phases. The two critical dE2F1 targets that appear to mediate this effect are the Cyclin E homolog CycE and String, a homolog of the CDC25 phosphatase which is required for entry into mitosis (Duronio and O'Farrell, 1995; Neufeld *et al.*, 1998). Overexpression of CycE shortens G_1 phase but this is compensated for by a prolonged S phase. The complete dE2F1 overexpression phenotype is only observed when both CycE and String are overexpressed.

Finally, as described above, *C. elegans efl-1* E2F was originally found to participate in two developmental processes that do not seem to relate directly to cell-cycle regulation. These are vulval cell fate specification and asymmetric segregation of early embryonic determinants (Ceol and Horvitz, 2001; Page *et al.*, 2001). Therefore, the functions of E2Fs *in vivo* are not necessarily restricted to regulating G_1 progression.

Cyclin D-dependent kinases

Cyclin D-dependent kinases, regulators of cell growth or proliferation?

Studies in mammalian tissue culture cells indicated that Cyclin D-dependent kinase activity is essential for entry into S-phase. Inactivation of Cyclin D-CDK4/6 complexes in tissue culture cells by antibody injections or overexpression of INK4 CDK inhibitors arrests cells in G_1

phase (Baldin *et al.*, 1993; Bruce *et al.*, 2000; Guan *et al.*, 1994; Koh *et al.*, 1995; Lukas *et al.*, 1995a; Lukas *et al.*, 1995b; Medema *et al.*, 1995). In both cases, the arrest is dependent on the function of pRb family members.

The most direct evidence that Cyclin D-CDK4/6 complexes are essential for entry into S-phase comes from studies in *C. elegans*. Mutation of the *C. elegans* genes *cyd-1* Cyclin D or *cdk-4* CDK4/6 results in an arrest of postembryonic cell divisions in G_1 phase (Boxem and van den Heuvel, 2001; Park and Krause). Similar analyses in mammals are complicated by the presence of three D-type cyclins and two kinase partners. Knockout mice lacking Cyclin D1, Cyclin D2 or CDK4 develop tissue specific abnormalities. This demonstrates that these genes are not essential for the development of mice into fertile adults (Fantl *et al.*, 1995; Rane *et al.*, 1999; Sicinski *et al.*, 1996; Sicinski *et al.*, 1995; Tsutsui *et al.*, 1999). Cyclin D1 knockout mice grow slowly and display several developmental defects. The retina of such mice shows a reduced cell number in all layers, although overall retinal architecture and function is initially unaffected (Fantl *et al.*, 1995; Sicinski *et al.*, 1995). Cyclin D1 deficient retinas undergo a wave of apoptosis between the second and fourth weeks after birth, and photoreceptor cells have a differentiation defect (Ma *et al.*, 1998). A second tissue affected by loss of Cyclin D1 is mammary epithelium. The dramatic expansion of mammary epithelium normally observed during pregnancy does not take place in Cyclin D1 mutant mice, even though steroid hormone and estrogen receptor levels are normal (Fantl *et al.*, 1995; Sicinski *et al.*, 1995). Finally, Cyclin D1 knockout mice have malformed jaws leading to unchecked growth of the incisor teeth (Fantl *et al.*, 1995), and a neurological defect, where the mutant mice retract their limbs toward the trunk when lifted by their tails, rather than extending them (Sicinski *et al.*, 1995).

Loss of Cyclin D2 also results in decreased cell numbers in two specific tissues. Few layers of somatic granulosa cells are found around the oocytes of Cyclin D2 deficient mice. These granulosa cells fail to proliferate in response to follicle stimulating hormone, which normally induces Cyclin D2 levels (Sicinski *et al.*, 1996). In addition, the testes of Cyclin D2 mutant mice do not grow to full size and the CNS contains less granule cells and almost no stellate interneurons (Huard *et al.*, 1999; Sicinski *et al.*, 1996). CDK4 deficient mice demonstrate tissue specific defects that appear to overlap partly with those observed in Cyclin D1 and D2 mutant mice. CDK4 mutant mice have a growth defect and fertility is reduced. Granulosa cell proliferation and differentiation are abnormal, and the males develop testicular hypotrophy (Rane *et al.*, 1999; Tsutsui *et al.*, 1999). In addition, loss of CDK4 causes insulin-dependent diabetes, likely due to a defect in the proliferation of β-islet cells in the pancreas (Rane *et al.*, 1999). Thus, the loss of D-type cyclins does affect cell proliferation to some extent. Redundancy

between the different cyclins and CDK4/6 genes may explain the relatively limited effects.

Surprisingly, *Cdk4*, the single *Drosophila* homolog of mammalian CDK4 and CDK6 kinases, is dispensable for cell division (Meyer *et al.*, 2000). Flies homozygous for a *Cdk4* null mutation develop into viable adults, although fertility is greatly reduced. Additionally, *Cdk4* mutant flies are smaller than their wild-type counterparts due to decreased cell numbers. Several lines of evidence indicate that the decreased size is caused by an overall slower cell growth rate. First, cells in *Cdk4* mutant wing disks have a lengthened cell cycle due to proportional increases in G_1, S and G_2 phases, rather than an increase in the G_1 population only (Meyer *et al.*, 2000). In addition, overexpression of the *Drosophila* Cyclin D homolog CycD and Cdk4 results in an increased number of cells, while cell size is either not affected or increased, depending on the tissue examined (Datar *et al.*, 2000). In contrast, overexpression of *Drosophila* E2F/DP or Cyclin E results in increased cell proliferation but decreased cell size. These results therefore indicate that in *Drosophila*, Cyclin D-CDK4/6 complexes affect cell growth rather than cell-cycle progression.

In *C. elegans*, the primary role of Cyclin D-dependent kinases appears to be the promotion of G_1 progression. Loss of *cyd-1* Cyclin D or *cdk-4* CDK4/6 blocks postembryonic cell divisions during G_1 phase, while cells continue to increase in size well past the time at which they initially arrested in G_1 phase (Boxem and van den Heuvel, 2001). In mice, two observations argue against the retinal and mammary defects of Cyclin D1 deficient mice being a secondary consequence of a defect in cell growth. First, Cyclin D1 mutant MEFs have normal growth rates upon serum stimulation of quiescent cells (Fantl *et al.*, 1995). Second, replacement of the Cyclin D1 gene with a human Cyclin E gene, or loss of $p27^{Kip1}$, which presumably results in increased Cyclin E/CDK2 activity, overcomes all of the defects observed in Cyclin D1 mice (Geng *et al.*, 1999; Geng *et al.*, 2001; Tong and Pollard, 2001). As Cyclin E/CDK2 kinases do not appear to regulate cell growth in mice or *Drosophila*, it is unlikely that the rescue by Cyclin E is based on mimicking a growth regulatory function of Cyclin D1.

One possible interpretation of the different effects of loss of Cyclin D in *Drosophila* compared with mammals and *C. elegans* is that Cyclin D-CDK4/6 complexes perform two functions. One is to phosphorylate pRb and thus stimulate S-phase entry. The other is to promote cell growth. Variation in the balance between these two functions between organisms would explain the different outward defects resulting from inactivation of Cyclin D-dependent kinases. However, to date no direct evidence exists to support such a model.

The role of Cyclin D-dependent kinases in tumor formation

A role for D-type cyclins and CDK4/6 in tumorigenesis is indicated by the presence of defects that increase Cyclin D-dependent kinase activity in many tumor cells (reviewed in Sherr, 1996). These include overexpression of cyclins and CDKs, a mutation that renders CDK4 insensitive to $p16^{INK4a}$, and loss of CDK4/6 inhibitors of the INK4a family. Such observations, however, provide only correlative evidence for a role in tumor formation for Cyclin D-CDK4/6 complexes. More direct evidence is provided by the observation that mice with increased Cyclin D levels are prone to tumor development. For example, expression of Cyclin D1 in mammary tissues leads to mammary adenocarcinomas (Wang *et al.*, 1994). In addition, mice deficient for the INK4 kinase inhibitor $p16^{INK4a}$, and mice with a targeted CDK4 alteration (Arg24Cys) that renders CDK4 insensitive to INK4 inhibitors, are both susceptible to tumor development, although to a lesser extent than for example mice deficient in $p14^{ARF}$ (Krimpenfort *et al.*, 2001; Sharpless *et al.*, 2001; Sotillo *et al.*, 2001).

In addition to the correlation between increased Cyclin D-CDK4/6 activity and increased tumor incidence, Cyclin D1 activity also appears to be essential for the formation of certain types of tumors. Robles *et al* reported that in three models of *ras*-mediated mouse skin tumorigenesis, the loss of Cyclin D1 reduces the efficiency of tumor formation (Robles *et al.*, 1998). In addition, Cyclin D1 is involved in oncogenic transformation by the *neu* proto-oncogene. Expression of *neu* increases Cyclin D1 levels *in vitro* and *in vivo*, and inhibition of Cyclin D1 blocks *neu*-induced transformation (Lee *et al.*, 2000). Moreover, Yu *et al* recently demonstrated that mammary tumor formation in mice by expression of the *ras* or *neu* oncogenes from the MMTV promoter is dependent on the presence of Cyclin D1 (Yu *et al.*, 2001). Loss of Cyclin D1, but not D2 or D3, fully protects against transformation by these two oncogenes. Protection against transformation correlates with the Cyclin D species induced by the oncogenic transformation, as *ras* and *neu* induce only cyclin D1. In contrast, tumorigenesis by expression of *myc* or w*nt-1*, which induce expression of Cyclin D2 and D3, was not affected by loss of Cyclin D1. Thus, the presence of functional Cyclin D1 is required for the formation of specific types of tumors, while other tumors may depend rather on Cyclin D2 or Cyclin D3.

The importance of pRb and Cip/Kip proteins as Cyclin D-CDK4/6 targets in vivo

In addition to phosphorylation of pRb, Cyclin D-dependent kinases are thought to have a second function: sequestration of the Cip/Kip family of CDK inhibitors ($p21^{Cip}$, $p27^{Kip1}$ and $p57^{Kip2}$), which inactivate CDK2 kinase complexes (Ekholm and Reed, 2000; Sherr, 2000). Although it was initially thought that Cip/Kip family members also negatively regulate CDK4 and CDK6, it was soon realized that Cyclin D-CDK4/6/$p27^{Kip1}$

complexes are still catalytically active, and that in fact most of the Cyclin D-CDK4/6 kinase activity in proliferating cells is found in complexes with Cip/Kip proteins (Blain *et al.*, 1997; Cheng *et al.*, 1999; LaBaer *et al.*, 1997; Soos *et al.*, 1996). Cip/Kip proteins were shown to facilitate assembly of active Cyclin D-CDK4/6 complexes and promote their nuclear localization (Cheng *et al.*, 1999; LaBaer *et al.*, 1997). Cyclin E/CDK2 kinase activity is required for entry into S-phase (Ekholm and Reed, 2000; Sherr, 1993). Sequestration of Cip/Kip family members by Cyclin D-CDK4/6 complexes thus might promote G_1/S progression by preventing Cip/Kip kinase inhibitors from inactivating Cyclin E/CDK2.

In addition to the functions described above, two CDK independent functions have also been reported for Cyclin D1. First, Cyclin D1 can bind to and activate the estrogen receptor, possibly by recruiting steroid receptor coactivators to the estrogen receptor in the absence of ligand (McMahon *et al.*, 1999; Neuman *et al.*, 1997; Zwijsen *et al.*, 1998). Activation of the estrogen receptor is normally required for the proliferation of mammary tissue during pregnancy, and ectopic activation of this receptor may play a role in breast tumor formation. In addition, Cyclin D1 can bind to the Myb-like transcription factor DMP1. This may prevent the transcription by DMP1 of $p19^{ARF}$, a protein that can induce a G_1 arrest by stabilizing p53 (Hirai and Sherr, 1996; Inoue *et al.*, 1999).

Several observations indicate that sequestering of Cip/Kip family members is important for the function of Cyclin D-dependent kinases *in vivo*. First, fibroblasts that lack expression of all Cip/Kip proteins continue to proliferate despite the absence of detectable Cyclin D-kinase activity, and are resistant to the G_1 arrest normally caused by expression of $p16^{INK4a}$ (Cheng *et al.*, 1999). Second, in mice deficient for the CDK4 gene, a higher percentage of Cyclin E is found in complexes with $p27^{Kip1}$ than in WT animals (Tsutsui *et al.*). Serum starved MEFs from these CDK4 knockout mice demonstrate a delayed entry into S-phase upon restimulation, and this delay can be overcome by loss of $p27^{Kip1}$ (Tsutsui *et al.*). Finally, the deletion of $p27^{Kip1}$ rescues essentially all phenotypes associated with the Cyclin D1 knockout (Geng *et al.*, 2001; Tong and Pollard, 2001). These include the defects in growth, proliferation and apoptosis in the retina and the abnormal proliferation of mammary epithelium during pregnancy.

These experiments clearly demonstrate an important interaction between Cyclin D1/CDK4 and $p27^{Kip1}$, and are consistent with the major function of Cyclin D-CDK4/6 complexes being the inactivation of Cip/Kip proteins. However, although the elevated CDK2 activity found in $p27^{Kip1}$ knockout mice is apparently enough to bypass CDK4/6 activity, such ectopic levels of CDK2 activity likely do not accurately reflect the level of CDK2 activity in wild-type mice. Therefore, these experiments do not prove conclusively that sequestering of Cip/Kip kinase inhibitors is the major function of Cyclin D-dependent kinases in mice.

In contrast to the data described above, results obtained in *Drosophila* and *C. elegans* support the idea that inactivation of pRb is an important function of Cyclin D-dependent kinases. As described above, deletion of *Drosophila Cdk4* still allows animals to develop to adulthood but severely reduces the fertility of females. Loss of the *Drosophila* Rb homolog RBF1 significantly restores this fertility defect, indicating that RBF1 acts downstream of Cdk4 (Meyer *et al.*, 2000). As described in more detail above, inactivation of the single *C. elegans* Rb family member also suppresses the defects found in *cyd-1* Cyclin D and *cdk-4* CDK4 mutants (Chapter 3). These results strongly suggest that *lin-35* Rb is an important target for *cyd-1* Cyclin D and *cdk-4* CDK4/6. Interestingly, the *cyd-1* and *cdk-4* mutant phenotypes are not fully suppressed by loss of *lin-35*, suggesting additional roles for *cyd-1* and *cdk-4* besides inactivation of *lin-35* Rb.

Clearly, the issue of which function of Cyclin D-dependent kinases is most important *in vivo* has not been settled, and the relative importance of each function may vary between organisms.

OUTLOOK

From the literature discussed in this chapter it is clear that considerable insight has been gained into the functions of the major G_1 regulatory pathways in animal development. However, important questions do remain about the different steps involved in cell-cycle entry. One such question is how extracellular signals connect to the basic cell cycle machinery. Perhaps the best understood example is regulation of Cyclin D-dependent kinase activity by the Ras/MAPK pathway, which affects Cyclin D1 production, stability and kinase activity (reviewed in Sherr, 2000). However, many other ways in which developmental signals can influence the cell cycle likely remain to be discovered. A second important question is what are the molecular mechanism involved in negative regulation of G_1 progression by pRb. Studies on the functions of histone deacetylase and Swi/Snf complexes have already implicated these complexes in cooperating with pRb. However, given the large number of proteins that have been reported to associate with pRb, extensive study will be required before we fully understand the ways in which pRb negatively regulates progression through G_1 phase.

Although Cyclin D-dependent kinases and Cyclin E-CDK2 can both phosphorylate pRb, the mechanism by which full inactivation of pRb is achieved remains unclear. Furthermore, it is not known what other targets of Cyclin D-CDK4/6 complexes are involved in controlling cell-cycle progression. Finally, what are the E2F target genes that are essential for G_1 progression, and what E2F target genes act outside of G_1 phase? In this thesis, I have made use of *C. elegans* to begin to address some of these questions. Chapter 4 describes the identification of novel genes that may cooperate with *lin-35* Rb in negatively regulating G_1 pro-

gression, and Chapter 5 describes the identification of a mutant allele that likely defines a critical target of *cyd-1* Cyclin D.

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Chapter 2

The *Caenorhabditis elegans* gene *ncc-1* encodes a *cdc2*-related kinase required for M phase in meiotic and mitotic cell divisions, but not for S phase

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ABSTRACT

We have identified six protein kinases that belong to the family of *cdc2*-related kinases in *Caenorhabditis elegans*. Results from RNA interference experiments indicate that at least one of these kinases is required for cell-cycle progression during meiosis and mitosis. This kinase, encoded by the *ncc-1* gene, is closely related to human *CDK1/Cdc2*, *CDK2* and *CDK3* and yeast *CDC28/cdc2*$^+$. We addressed whether *ncc-1* acts to promote passage through a single transition or multiple transitions in the cell cycle, analogous to *CDKs* in vertebrates or yeasts, respectively. We isolated five recessive *ncc-1* mutations in a genetic screen for mutants that resemble larval arrested *ncc-1(RNAi)* animals. Our results indicate that maternal *ncc-1* product is sufficient for embryogenesis, and that zygotic expression is required for cell divisions during larval development. Cells that form the postembryonic lineages in wild-type animals do not enter mitosis in *ncc-1* mutants, as indicated by lack of chromosome condensation and nuclear envelope breakdown. However, progression through G_1 and S phase appears unaffected, as revealed by expression of ribonucleotide reductase, incorporation of BrdU and DNA quantitation. Our results indicate that *C. elegans* uses multiple CDKs to regulate cell-cycle transitions and that *ncc-1* is the *C. elegans* ortholog of *CDK1/Cdc2* in other metazoans, required for M phase in meiotic as well as mitotic cell cycles.

INTRODUCTION

A fundamental question in biology is how cell division, growth and differentiation are coordinately regulated during development of multicellular organisms. This coordination likely involves connections between developmental signals and the cell-cycle machinery. Cyclin-dependent kinases (CDKs) have been identified as key components of the cell-cycle machinery in all eukaryotes studied (Forsburg and Nurse, 1991; Nigg, 1995; Norbury and Nurse, 1992). In yeasts, progression through the cell cycle is controlled by a single CDK, encoded by the *CDC28* and *cdc2*$^+$ genes in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, respectively. The CDC28/cdc2 catalytic subunit requires association with a cyclin regulatory partner for kinase activity, and different cyclins are involved in progression through G_1, S and M phase. Regulation of cell-cycle progression is more complex in multicellular organisms; a large family of kinases related to CDC28/cdc2 has been identified in metazoans and several of these kinases have been implicated in cell-cycle regulation (reviewed by Nigg, 1995). In addition, various cyclins are expressed during distinct phases of the cell cycle in metazoans. Thus, progression through the cell cycle in higher eukaryotes is

thought to require successive activation of different CDKs, regulated in part by transient associations with distinct cyclins. Other levels of regulation include association with kinase inhibitors and activating as well as inactivating phosphorylations.

When vertebrate cells enter a division cycle, the first CDKs to become activated are CDK4 and/or CDK6 in association with D-type cyclins (Sherr, 1994; Sherr, 1996). Late in G_1, CDK2 is activated and can be found associated first with cyclin E and subsequently cyclin A (Tsai *et al.*, 1993a). Kinase activity of CDK1/Cdc2 in combination with A- and B-type cyclins peaks at the G_2/M transition (Draetta and Beach, 1988; Tsai *et al.*, 1993a). Several lines of evidence indicate that these CDKs regulate specific cell-cycle transitions. Extracellular signals, such as growth factors, influence expression levels of D-type cyclins and association with CDK4 and CDK6 (Matsushime *et al.*, 1994; Matsushime *et al.*, 1991). Ectopic expression of cyclin D, as well as cyclin E, shortens G_1 phase and accelerates entry into S phase (Ohtsubo and Roberts, 1993; Quelle *et al.*, 1993). Inhibition of either cyclin A activity or CDK2 activity inhibits DNA replication (Fang and Newport, 1991; Girard *et al.*, 1991; Pagano *et al.*, 1993; Tsai *et al.*, 1993a; van den Heuvel and Harlow, 1993), while inhibition of CDK1/Cdc2 prevents entry into mitosis (Fang and Newport, 1991; Riabowol *et al.*, 1989; Th'ng *et al.*, 1990; van den Heuvel and Harlow, 1993). The function of several other cdc2-related kinases is currently either unknown or, for CDK5, appears unrelated to cell-cycle progression (Chae *et al.*, 1997; Gilmore *et al.*, 1998; Nikolic *et al.*, 1996). Thus far, genetic analysis of *CDK* loss-of-function mutations has been limited in multicellular organisms. A family of cdc2-related kinases has been identified in *Drosophila* and one of these kinases, Dmcdc2, has been implicated in regulation of mitosis and inhibition of DNA replication (Hayashi, 1996; Sauer *et al.*, 1996; Stern *et al.*, 1993). A second kinase, Dmcdc2c, appears active during S phase (Lehner and O Farrell, 1990).

The nematode *C. elegans* is uniquely suited as an animal model to study developmental control of cell division. The transparency of these animals allows monitoring of cell divisions in living animals. In addition, the near invariance of the cell lineage has allowed a precise description of the time of division for every somatic cell (Sulston and Horvitz, 1977; Sulston *et al.*, 1983). Combined with effective genetics, these characteristics allow efficient isolation and detailed examination of cell-cycle mutants.

Genetic studies have already revealed several genetic pathways that regulate postembryonic cell divisions. For instance, pathways that involve TGF-β and insulin-related signaling cascades control entry into and exit from a developmentally arrested dauer stage (Massague, 1998; Riddle *et al.*, 1997). In addition, a pathway of heterochronic genes controls the timing of cell division in several postembryonic lineages (reviewed by Slack and Ruvkun, 1997). Furthermore, a Notch-like sig-

naling pathway that includes the *lag-2* ligand and *glp-1* receptor controls the switch between mitotic versus meiotic division in the germline (Francis *et al.*, 1995; Kadyk and Kimble, 1998; Kimble and Simpson, 1997). The targets of such developmental signaling pathways likely include components of the general cell-cycle machinery, which may be regulated through intermediates such as *cki-1*, a developmentally regulated CDK inhibitor of the CIP/KIP family (Hong *et al.*, 1998).

To be able to explore connections between developmental signals and the cell-cycle machinery, we have initiated analysis of the cyclin-dependent kinases present in *C. elegans*. We have identified six putative kinases that share extensive homology with cdc2-related kinases in higher eukaryotes. RNA-interference of only one of these kinases caused complete arrest of cell division. A cDNA clone of this kinase, named *ncc-1* for nematode cell cycle, was previously identified and shown to complement the *CDC28-1N* mutation in *S. cerevisiae* (Mori *et al.*, 1994). Here we show that *ncc-1* is required for progression through M phase in meiotic and mitotic cell cycles, but not for G_1 and S phase progression. Thus, *ncc-1* performs a role similar to *CDK1/Cdc2* in higher eukaryotes.

RESULTS

Identification of Cdc2 family members in *C. elegans*

Using several molecular approaches, we obtained cDNA clones from three *C. elegans* cdc2-related kinases (Materials and Methods and Fig. 1A). The kinase most closely related to human CDK1 (64% amino acid identity) was identified previously and named *ncc-1* (Mori *et al.*, 1994). The predicted NCC-1 protein of 332 amino acids diverges from other CDK1 and CDK2 kinases at two striking positions: it has an N-terminal extension of 18 amino acids and contains an Ile-to-Val substitution in the highly conserved PSTAIRE region of the protein that is involved in cyclin binding (Jeffrey *et al.*, 1995). A second kinase gene encodes a member of the PCTAIRE subfamily and was therefore named *pct-1*. Within the catalytic core, the predicted PCT-1 protein of 577 amino acids shares ~80% identity with any one of three human PCTAIRE kinases. In addition, PCT-1 has N-terminal and C-terminal extensions that are less conserved but similar in size to the human kinases. A third *C. elegans* kinase is a close homolog of human CDK5 (74% amino-acid identity) and was named *cdk-5*. For each of these three kinases we sequenced the longest cDNAs and determined the genomic structure (Fig. 1A).

Three additional kinases that are more distantly related to Cdc2 were identified in BLAST homology searches (Table 1). The predicted gene H06A10.1 encodes a kinase that is most related to CDK4 and CDK6. K03E5.3 encodes a kinase that is 42%, 43% and 41% identical to

Table 1. *cdc2*-related kinases in C.*elegans*

C. elegans gene	Human homolog	Amino acid identity (%)	RNAi phenotype
ncc-1	CDK1	64	One-cell stage embryonic arrest. In the first 12 hours after injection, up to 10 progeny arrest cell division at the L1 stage.
	CDK2	59	
	CDK3	60	
K03E5.3	CDK1	42	Embryonic lethal, larval arrest without growth, larval arrest with growth, sterile animals with protruding vulva (see text).
	CDK2	43	
	CDK3	41	
H06A10.1	CDK4	37	Variable cell division defects in larval development*.
	CDK6	33	
cdk-5	CDK5	74	No phenotype detected.
B0285.1	Ched	47	Late L3 or early L4 arrest.
	CDK2	41	
	CDK3	41	
pct-1	Pctaire-1	52	No phenotype detected.
	Pctaire-2	50	
	Pctaire-3	60	

*M. Park and M. Krause, personal communication.

human CDK1, CDK2 and CDK3, respectively. Finally, the predicted product of B0285.1 shares 47% amino acid identity with human CHED kinase, and 41% with CDK2 and CDK3. As the sequencing of the *C. elegans* genome has been completed (The *C. elegans* Sequencing Consortium, 1998), we can conclude that these are the *C. elegans* kinases that are most closely related to members of the CDK1/2/3, CDK5, CDK4/6 and PCTAIRE subfamilies of cdc2-related kinases.

***ncc-1* is a candidate cell-cycle regulator**

We used RNA interference (RNAi) to determine which of these kinases may be involved in regulation of the *C. elegans* cell cycle. RNAi has been shown to cause specific loss-of-function phenotypes for many different genes in *C. elegans*, although the mechanism of action is not understood (Guo and Kemphues, 1996; Tabara *et al.*, 1998). Double-stranded RNA (dsRNA) is at least one order of magnitude more potent in interfering activity than either single strand (Fire *et al.*, 1998). We transcribed both strands of each of the six kinases *in vitro* and injected dsRNAs into wild-type hermaphrodites. Injection of *ncc-1* dsRNA caused a completely penetrant embryonic arrest at the one cell stage in all progeny produced after 12 hours post-injection. In addition, some of the earlier progeny arrested during larval development (31 larval mutants from 10 injected animals). These arrested larvae were uncoordinated, sterile, slightly dumpy and had disproportionally large head regions. Examina-

tion by Nomarski microscopy revealed that no or very few cells in the postembryonic lineages had divided. Most striking was the absence of any division of the blast cells Z1 to Z4 that normally form the somatic gonad and germ-line. These gonad precursor cells divide multiple times in several *C. elegans* cell-cycle mutants, including *lin-5* and *lin-6* mutants (our unpublished observations, Sulston and Horvitz, 1981). Both the embryonic and L1-arrest phenotypes implicated *ncc-1* as an important cell-cycle regulator.

RNAi for two *Cdc2* related genes, *cdk-5* and *pct-1*, did not result in any apparent phenotypes (data not shown). RNAi for each of the remaining three *cdc2*-related genes caused obvious developmental defects but not a strict arrest of cell division. A clear but highly variable effect was observed following dsRNA injection of K03E5.3: the progeny arrested either as embryos (12%, n=69), early larvae (17%, n=98), late larvae (9%, n=50), sterile adults (39%, n=228) or developed to wild-type adults (24%, n=132). Progeny from B0285.1 dsRNA-injected animals arrested late in the third larval stage (L3) or early L4 stage. Most cell divisions have been completed at this stage with the exception of germ-line divisions. Finally, H06A10.1 RNAi resulted in slightly abnormal progeny but no apparent cell-cycle defects. Variable defects in cell division have been observed to result from H06A10.1 RNAi by M. Park and M. Krause (personal communication). Redundancy between different kinases might mask individual gene functions. However, injection of a mix of dsRNAs from K03E5.3, F18H3.5, B0285.1 and *cdk-5* did not cause stronger defects: the phenotype observed closely mimicked that of K03E5.3 RNAi. Thus, based on these RNAi results, *ncc-1* is the best candidate for a general cell-cycle kinase in *C. elegans*.

NCC-1 is closely related to yeast CDC28/cdc2 as well as mammalian CDK1 and CDK2. Therefore, *ncc-1* could act analogously to *CDC28/cdc2*$^{+}$

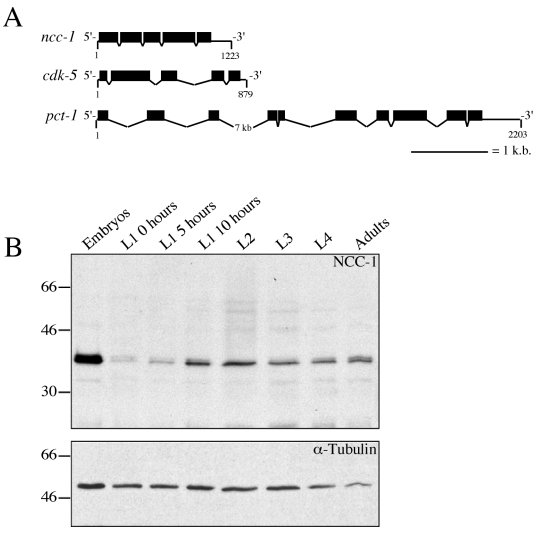

Fig. 1. (A) Gene structure of the *C. elegans cdc2*-related genes *ncc-1*, *cdk-5* and *pct-1*. Gene structures were determined by Southern blotting and DNA sequencing. Structures have been confirmed by the *C. elegans* sequencing project. (B) NCC-1 protein levels correlate with cell divisions. Protein lysates of synchronized wild-type animal populations were immunoblotted and probed with anti-PSTAIRE antibodies. α-tubulin protein levels were determined as a loading control.

in yeasts and regulate multiple transitions in the cell cycle. Alternatively, *ncc-1* could play a role similar to one of the human kinases, regulating either G_1/S progression or mitosis. To distinguish between these possibilities, we determined the requirements of *ncc-1* in the *C. elegans* cell cycle.

NCC-1 protein expression correlates with cell division and is abolished by RNAi

If *ncc-1* is required for cell division in general, NCC-1 protein is expected to be present in dividing cells. NCC-1 protein levels were found to correlate with the number of cell divisions during various stages of development, as was shown by western blot analysis (Fig. 1B). The highest NCC-1 levels were detected in embryos and the lowest levels in L1 larvae that were arrested by food deprivation. Upon release from L1 arrest, and resumption of cell division, NCC-1 protein levels were found to increase.

We used two different antibodies to detect the localization of NCC-1: a monoclonal antibody that recognizes the PSTAIRE peptide and an antiserum raised against a C-terminal NCC-1 peptide. Identical staining patterns were obtained with both antibodies. NCC-1 was found in the nucleus of oocytes and embryonic cells and, in addition, diffusely localized in the cytoplasm (Fig. 2A-F). During growth of oocytes in the proximal gonad, NCC-1 levels gradually increase (Fig. 2A,H). Following nuclear membrane breakdown, staining was diffuse in mitotic cells (Fig. 2D). NCC-1 appeared excluded from the DNA in metaphase and, to a lesser extent, in anaphase cells. NCC-1 was not detectable in oocytes or embryos of RNAi-treated animals (Fig. 2G,I). This result confirms the staining specificity and supports that *ncc-1(RNAi)* results in strongly reduced NCC-1 protein levels.

***ncc-1* is required for meiotic maturation**

To determine the cause of arrest in *ncc-1(RNAi)* embryos, we analyzed gametogenesis and early embryogenesis in the injected hermaphrodites. In wild-type animals, germ-precursor nuclei are produced by mitotic divisions in the distal ends of the syncytial gonad (Kimble and White, 1981). As these nuclei move further from the distal tip cell, they exit the mitotic cycle, initiate meiosis and progress through pachytene of meiotic prophase I. Oocyte formation begins when germ nuclei reach the flexure in the U-shaped gonad; they exit from pachytene, cellularize, continue to enlarge and progress to diakinesis of Meiosis I. Shortly before fertilization, meiotic maturation and nuclear envelope degradation are initiated. Mature oocytes ovulate by entering the spermatheca at the proximal end of the gonad. Meiotic maturation is completed after fertilization and two polar bodies are expelled from the fertilized egg. The maternal pronucleus subsequently migrates towards the paternal pronucleus, while the egg undergoes pseudocleavage. The

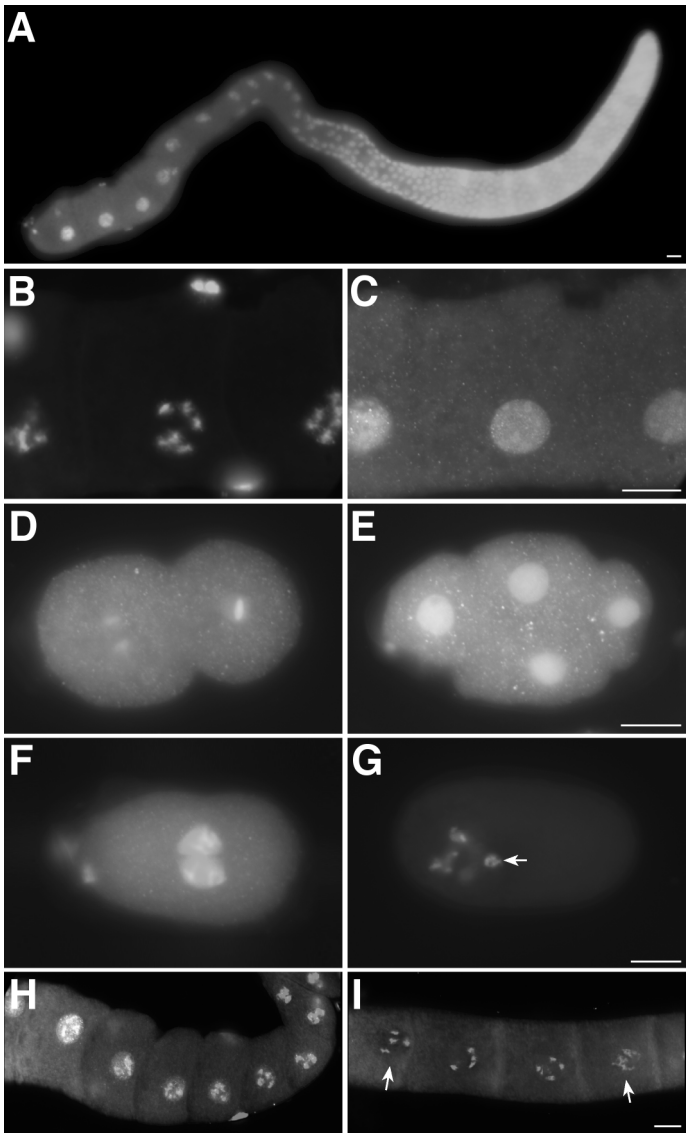

Fig. 2. NCC-1 protein localization in wild-type and *ncc-1(RNAi)* germ cells and embryos. (A) Nuclear NCC-1 expression levels increase during oocyte development (from right to left). A close-up of oocytes is shown in which DNA (B) and NCC-1 (C) are stained. NCC-1 localization is cytoplasmic during mitosis. (D) a wild-type 2 cell embryo in which the AB cell is in anaphase (left) and the P1 cell in metaphase (right). (E) Nuclear and cytoplasmic NCC-1 localization in a 4 cell wild-type embryo. (F,G) Comparison of NCC-1 staining in wild-type embryo (F) and *ncc-1(RNAi)* embryo (G). Exposure times and processing were identical for both images. NCC-1 staining is absent in *ncc-1(RNAi)* embryos. An arrow indicates condensed DNA in the paternal pronucleus. (H,I) NCC-1 and DNA (PI) staining in wild -type and *ncc-1(RNAi)* oocytes. NCC-1 accumulates in nuclei of developing oocytes (from right to left) in the wild-type (H) but not in dsRNA-injected gonads (I). Note that chromosomes fully condense (compare the earlier (right) and later (left) nuclei indicated) to the diakinesis state in oocytes following RNAi treatment. Scale bars approx. 10 μm.

two pronuclei meet in the posterior of the egg, migrate to the middle and initiate a first mitotic division.

We examined the consequences of *ncc-1* dsRNA injection on these maturation and fertilization events in live animals by Nomarski microscopy. Two types of abnormalities were apparent: at the distal ends of the gonad fewer germ cells were produced (see below) and at the proximal ends many of the events that follow ovulation and fertilization of oocytes were aberrant (Table 2). Gonads were examined at various time points following dsRNA injection by Nomarski microscopy of living animals and fluorescence microscopy of fixed worms stained with DAPI for analysis of DNA organization. Formation and growth of oocytes was normal. The morphology and progression from pachytene to late stage oocytes, with six fully condensed bivalents in typical diakinesis arrangement, was also normal (Fig. 2I). Based on the position of the most distal oocyte with fully condensed chromosomes, relative to the flexure in the gonad, progress through pachytene appeared slightly delayed in about half of the injected animals (data not shown).

The first apparent defect was abnormal breakdown of the nuclear membrane in the mature oocyte, which required about 20 minutes to complete instead of 5 minutes in the wild type. Cytological analysis demonstrated that the transition from diakinesis to metaphase of meiosis I failed to occur as chromosomes did not congress to form a metaphase plate. No polar bodies were observed consistent with a complete failure of the meiotic divisions (Fig. 2G). However, fertilization did trigger formation of an eggshell. The maternal pronucleus enlarged and became about twice the wild-type size. Migration of the maternal pronucleus toward the paternal pronucleus was initiated but meeting of the nuclei was delayed and pseudocleavage did not occur (Table 2). The chromosomes in the paternal pronucleus failed to decondense and this nucleus remained small (Fig. 2G). Meeting of the pronuclei was followed by sta-

Table 2. Timing of events following ovulation in *ncc-1(RNAi)* embryos

	N2 (n=5)*	*ncc-1* RNAi (n=6)
Entry to uterus	5.3±0.5	5.8±0.8
Appearance of paternal pronucleus	31.8±3.9	28.5±7.9
Pseudocleavage	33.4±4.8	does not occur
Appearance of maternal pronucleus	33.5±4.6	29.3±5.5
Pronuclear meeting	39.5±4.7	58.6±2.4
Nuclear envelope breakdown	45.2±4.8	does not occur
Onset of cytokinesis	49.7±5.9	does not occur
Completion of cell division	56.2±6.7	does not occur

Time is in minutes measured from ovulation.

*Similar results have been described by (Kirby *et al.*, 1990; McCarter *et al.*, 1998; Rose *et al.*, 1997).

ble arrest: the nuclear membranes did not break down and mitosis did not occur. By immunostaining with tubulin-specific antibodies, we detected a bipolar spindle around the maternal nucleus in about half of the embryos (data not shown). We cannot exclude that formation of this spindle is dependent on residual activity of *ncc-1* in the *ncc-1(RNAi)* embryos (see Discussion).

The earliest defect seen in *ncc-1(RNAi)* animals is lack of meiotic maturation. Thus, these results suggest that *ncc-1* is required to promote meiotic maturation. The subsequent abnormalities may be secondary consequences of the meiotic maturation defect.

***ncc-1* is required for mitotic divisions in the germ-line**

We noticed that *ncc-1* dsRNA-injected animals produced fewer oocytes than wild-type hermaphrodites. Upon closer examination, the number of germ cells was found to decrease gradually over time in *ncc-1* RNA-injected animals. This reduction was first apparent in the mitotic region of the gonad arms, starting at around 12 hours after injection, at 20°C. The gonad was essentially empty by day three to four. During the same period, control injected animals did not show a noticeable change in the number of germ cells or general appearance of the gonad.

The germ precursor nuclei that were present in the distal region at 20 hours after injection were enlarged (Fig. 3B). Mitotic cells were not observed in gonads fixed and stained with DAPI. As metaphase cells are not always unambiguously detected in wild-type gonads, we also examined the presence of a mitotic phosphorylated epitope. Chromosome condensation in mitosis has been described to coincide with phosphorylation of histone H3 on Ser-10, which forms part of a consensus Cdc2-phosphorylation site (Ajiro *et al.*, 1996; Hendzel *et al.*, 1997). A rabbit polyclonal antiserum raised against a phosphorylated H3 peptide also recognizes a mitotic phospho-epitope in *C. elegans* (Kadyk and Kimble, 1998; Lieb *et al.*, 1998). We immunostained *ncc-1* dsRNA-injected and control injected animals 20 hours after injection to examine the presence of the phosphorylated histone H3 epitope. Staining was absent

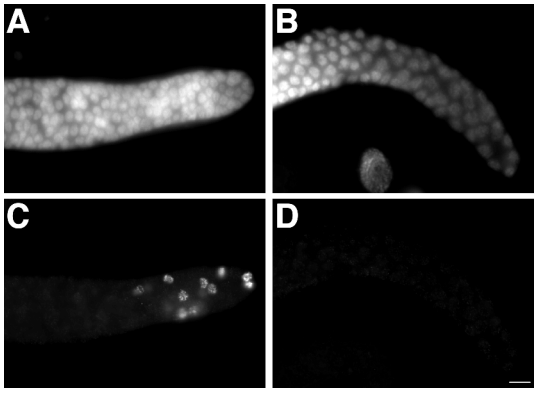

Fig. 3. Arrest of mitotic divisions in the germline by *ncc-1(RNAi)*. Germ cells in the distal, mitotic region of the gonad were stained with the DNA stain DAPI (A,B) and antibodies recognizing phosphorylated histone H3 (C,D). Wild-type germ cells in mitosis stain with the phosphorylated histone H3 antibody (C), whereas staining is not detected following *ncc-1* RNAi (D). Scale bar approx. 10 μm.

in *ncc-1* RNA-injected germlines whereas control injected animals stained similarly to wild type (Fig. 3C,D). NCC-1 could directly or indirectly cause phosphorylation of histone H3, or be required at an earlier step in germ cell division. In either case, when taken together, these observations indicate that *ncc-1* is required for mitotic divisions in the distal part of the gonad.

Isolation of *ncc-1* mutants based on the larval *ncc-1(RNAi)*phenotype

To further characterize the role of *ncc-1* in cell division, we isolated *ncc-1* mutations in a genetic screen. The larval arrested *ncc-1(RNAi)* animals may have received functional *ncc-1* maternal product yet fail zygotic expression. Based on this interpretation, we screened for uncoordinated, sterile mutants with disproportionally large head regions in the F2 progeny of ethylmethanesulfonate (EMS) treated animals, in order to isolate *ncc-1* mutants.

In a semi-clonal screen of about 22000 haploid genomes, we obtained 21 independent recessive mutations that caused uncoordinated, sterile, large head phenotypes and defects in cell division during the first larval stage. Based on standard complementation assays and genetic mapping, 14 of these mutations likely affect the same gene. We characterized 4 of these mutations in detail. In addition, we also characterized *n3064* which displays a very similar phenotype and was isolated in a previous screen for potential cell-cycle mutants (S. v.d.H. and H. R. Horvitz, unpublished results). All five of these mutations mapped in close proximity to *ncc-1* and failed to complement *nDf40*, a deletion that removes the *ncc-1* locus. In addition, DNA sequencing revealed mutations within the *ncc-1* ORF in all five mutants (Fig. 4). The mutant alleles all contain G-C to A-T transitions, the type of mutation most commonly induced by EMS. The best candidate null allele is *he5*. This allele contains a deletion of 131bp, that removes the entire PSTAIRE domain, as well as a nonsense mutation that should cause a truncated product (Fig. 4). Allele *he26* also contains a nonsense mutation and encodes a product that lacks the C-terminal 265 out of 332 kinase residues. The other three alleles, *he24*, *n3064* and *he25*, contain missense mutations at positions 75, 191 and 214, respectively, replacing negatively charged glutamates by positively charged lysine residues. Each mutation affects a residue that is highly conserved within the Cdc2 family, in *he24* and *he25*, or in all protein kinases, in *n3064* (Fig. 4).

***ncc-1* is required for postembryonic cell divisions**

We characterized the nature of the *ncc-1* alleles and cell-cycle phenotypes in more detail. As homozygous mutants are fully penetrant sterile, the *ncc-1* mutations were maintained in heterozygous strains. Derived from heterozygous (*ncc-1*/+) mothers, *ncc-1* mutant progeny completed embryogenesis, presumably as a result of maternal gene function, but displayed no cell divisions after hatching. The percentage mutant prog-

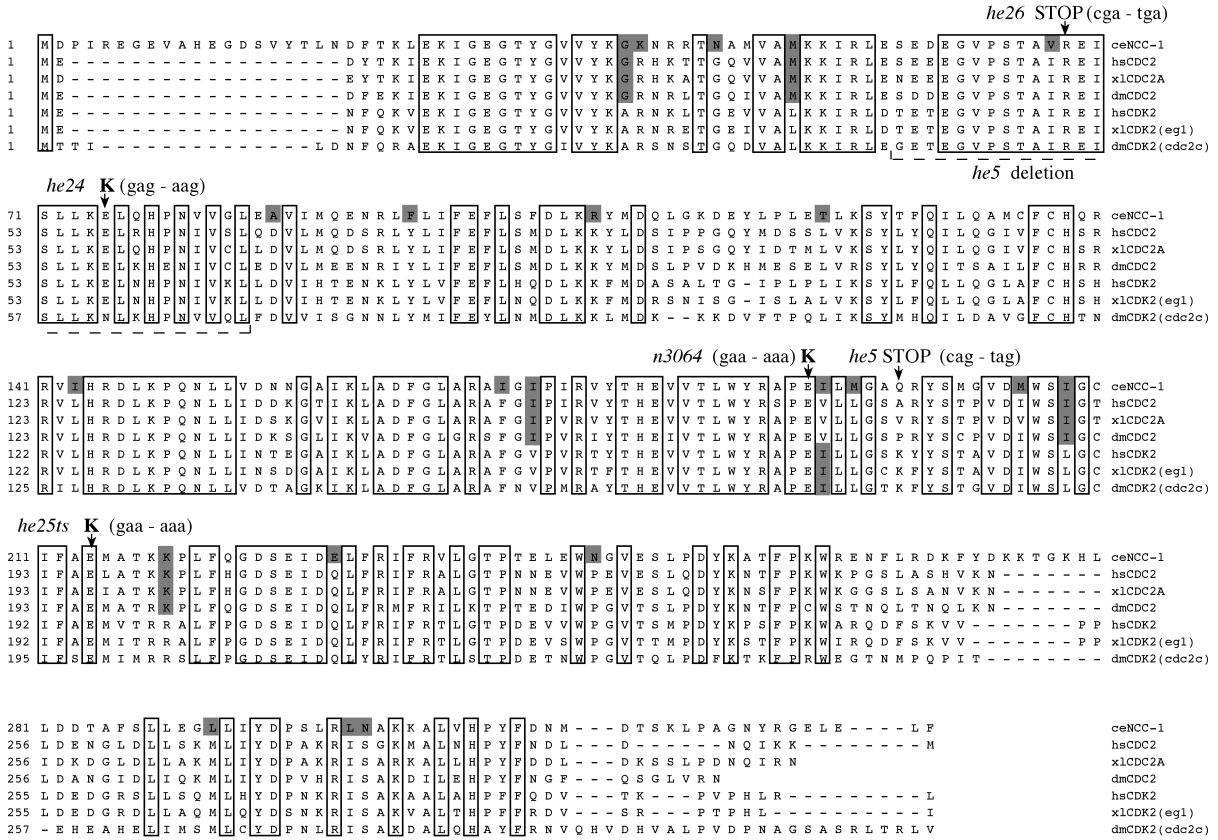

Fig. 4. Alignment of NCC-1 protein with CDK1/Cdc2 and CDK2 kinases from human, *Xenopus laevis* and *Drosophila melanogaster*. Boxed regions indicate regions of amino acid identity. Shaded small squares indicate residues that are conserved in Cdc2 and CDK2 kinases but not in NCC-1. Shaded rectangles indicate residues conserved between NCC-1 and Cdc2 kinases, but distinct in CDK2 kinases. One shaded box (near *n3064* mutation) indicates a residue shared between NCC-1 and CDK2 kinases, but not present in Cdc2 kinases.

eny was close to 25% for all mutations, confirming the recessive nature of the alleles (Table 3). At 25°C, homozygous mutants and trans-heterozygotes with *nDf40* were phenotypically indistinguishable. Thus, all five mutations cause fully penetrant strong loss-of-function phenotypes at 25°C. Similar results were obtained at 15°C for all alleles except *he25*. Homozygous *he25* mutants were of more wild-type appearance at 15°C, although shorter and fully penetrant sterile. Based on our genetic and molecular characterizations, 4 of the 5 alleles are strong loss-of-function or null mutations, whereas *he25* causes incomplete loss of function at 15°C.

Nomarski microscopy observations indicated that all blast cells that give rise to the postembryonic lineages in wild-type animals are present in *ncc-1* mutants. However, these cells did not initiate mitotic divisions, with the possible exception of a rare division of an intestinal nucleus. Examination of fixed and DNA-stained animals confirmed these findings (Table 3). We quantified the cell-cycle defects for each of the mutations in three postembryonic lineages, by examining the first divisions of the ventral cord precursor cells (P), the intestinal cells (I) and the precursor cells of the somatic gonad and germline (Z1, Z2, Z3 and Z4). In wild-type animals, these cells start to divide about halfway through L1 (P and Z1-4) or late in L1 stage (I). All five mutations caused very similar defects in cell division. Not a single division of cells in the gonad primordium or P1-10 was observed in 20 animals exam-

Table 3. Characterization of *ncc-1* alleles

Parental genotype	% larval arrest of progeny (*n*)	Postembryonic blast cell divisions (no.)		
		P1-P10 (*n*=20)	I (*n*=20)	Z1-Z4 (*n*=20)
Wild type	0.0 (>2000)	200	263	80
he5/+	23.2 (1393)	0	2	0
he24/+	25.0 (1106)	0	9	0
he25/+ at 15°C	26.4 (1346)	ND	ND	ND
he25/+ at 25°C	25.6 (1047)	0	11	0
he26/+	25.4 (1405)	0	7	0
n3064/+	26.9 (1039)	0	2	0

Total progeny of four *ncc-1/+* heterozygotes was counted to determine percentage larval arrest. Divisions of P-cells and I cells were scored in mutants fixed and stained with the DNA stain PI. Divisions of P11 and P12 could not be unambiguously determined and are therefore excluded. For P-cell divisions, animals were fixed 24 hours at 15°C after release from an L1 arrest, except *ncc-1(he25)* mutants, which were fixed after 9 hours at 25°C. For I-cell divisions, animals were fixed as adults. Z-cell divisions were examined *in vivo* by Nomarski microscopy, 28 hours after release from L1 arrest at 20°C, except *ncc-1(he25)* mutants, which were scored 24 hours at 25°C after release from the L1 arrest. n=number of animals examined.

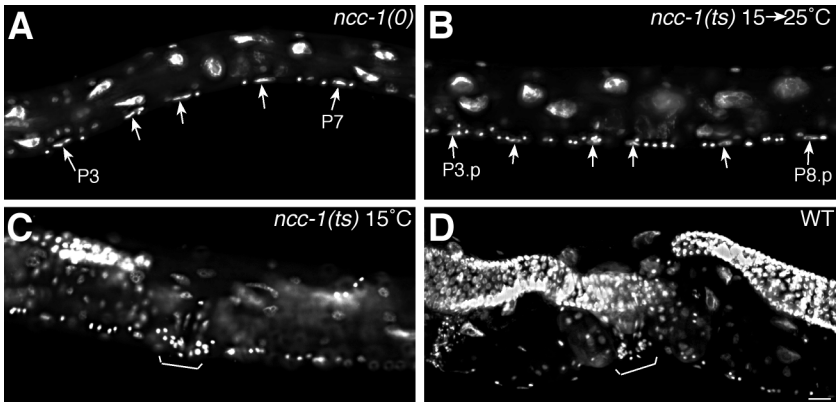

Fig. 5. (A,B) *ncc-1* is required for postembryonic blast cell divisions. DNA is stained with propidium iodide, all panels show animals of similar age (adults). (A) An *ncc-1(he5)* mutant, in which P cell divisions have not occured. P3-P7 are indicated by arrows. (B) A temperature sensitive *ncc-1(he25)* mutant shifted from 15°C to 25°C in the third larval stage, which caused the Pn.p divisions to fail. Undivided P3.p-P8.p cells are indicated by arrows. (C) An *ncc-1(he25)* animal grown at 15°C, which allowed most divisions in the P lineages as well as formation of a vulva (D) Wild-type adult. The bracket indicates the position of the vulva. Scale bar approx. 10 μm.

ined (Table 3). Several observations indicate that *ncc-1* is directly required for cell division and that the lack of division was not caused by sickness or lethality. The *ncc-1* mutants were viable and continued to grow to 80% of the wild-type size (the length of young adult *ncc-1* and wild-type animals was 0.73±0.04 mm and 0.91±0.08 mm, respectively). In addition, the precursor cells of the ventral nerve cord migrated at the normal time from a more lateral to a ventral position (data not shown).

Requirement for *ncc-1* is not restricted to the first postembryonic or L1 divisions, as was shown in *he25ts* animals. At 15°C, the P cells undergo extensive divisions during the L1 stage in *he25ts* mutants and the P3.p to P8.p (Pn.p) daughters divide in late L3 to form the vulva (Fig. 5C). All P cells failed to divide in *he25* animals shifted to 25°C during the early L1 stage, thereby resembling *ncc-1(0)* animals (Table 3). In contrast, temperature shift during the L2 or L3 stages allowed the P cells to divide during L1 but prevented division of the Pn.p daughters (Fig. 5B).

These results are consistent with a role for *ncc-1* as a general cell-cycle regulator, which is in agreement with the high degree of identity between NCC-1 and the CDK1 and CDK2 kinases in other species (Fig. 4).

ncc-1 is required for mitosis, but not for DNA replication

We observed that the precursor cells of the postembryonic lineages did not initiate chromosome condensation or nuclear envelope breakdown in *ncc-1* mutants. Apparently, the cells arrest prior to entry into mitosis,

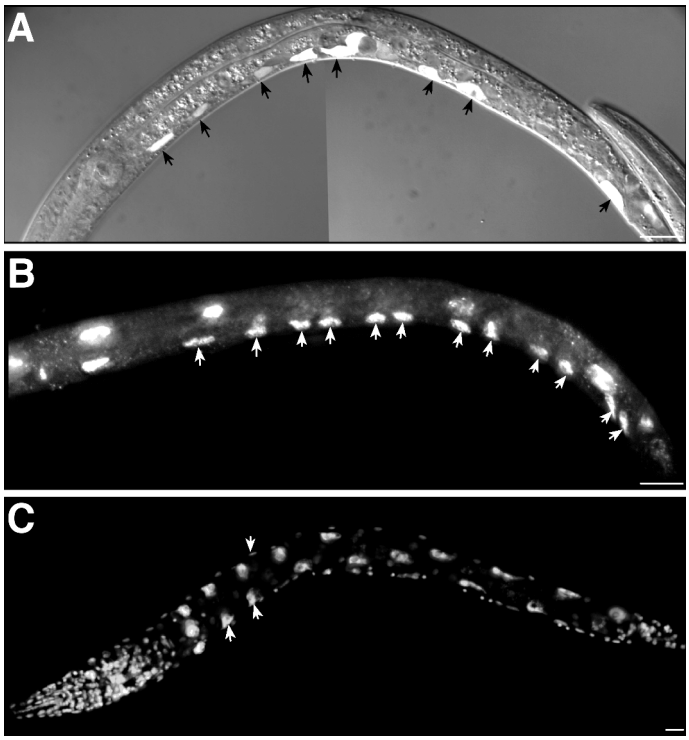

Fig. 6. *ncc-1* mutants complete G_1 and S phase. (A) GFP expressed from the *rnr::GFP* reporter construct in an *ncc-1(n3064)* mutant. Several P-cells expressing rnr::GFP are indicated. (B) BrdU incorporation in an *ncc-1(he5)* mutant. Incorporation of BrdU can be detected in all P-cells (arrows). (C) DNA stained with propidium iodide in adult *he24* animal. Arrows indicate body wall muscle cell (2n, top) and polyploid intestinal cells (32n, bottom). Scale bars approx. 10 μm.

yet this arrest could occur in G_1, S or G_2 phase. We examined whether cells in *ncc-1* mutants initiate S phase to determine the point of arrest.

As a first assay, we used an S phase reporter that expresses the green fluorescent protein (GFP) under the control of a *C. elegans* ribonucleotide reductase (*rnr*) promoter. Expression of *rnr* tightly correlates with S phase in other eukaryotes (Fernandez-Sarabia and Fantes, 1990). In addition, rnr::GFP expression in transgenic worms that contain the integrated *rnr::gfp* reporter coincides with the previously reported time of S phase (Hong *et al.*, 1998). We examined GFP expression in L1 larvae that were double homozygous for the *ncc-1(n3064)* mutation and integrated *rnr::gfp*. Wild-type animals and *ncc-1* mutants were found to express rnr::GFP at the same time and in the same cell types (Fig. 6A). Thus, blast cells of the postembryonic lineages complete G_1 and appear to become competent for S phase in *ncc-1* mutants.

To directly address whether *ncc-1* mutants synthesize DNA, we examined incorporation of the thymidine analog BrdU. Progeny from animals heterozygous for *he5*, *he26* or *n3064* were incubated with BrdU

from 3 to 9 hours of L1 development at 25°C and subsequently stained with anti-BrdU antibodies. In wild-type animals as well as *ncc-1* mutants, BrdU incorporation was detected in all cells that were previously reported to undergo S phase during this developmental phase (Hedgecock and White, 1985). For example, BrdU staining was detected in the ventral cord precursor cells W and P1-P12 (Fig. 6B), and these cells divided in the wild-type but not in *ncc-1* mutants. Other cells that stained strongly with BrdU in wild-type as well as homozygous *ncc-1* animals include the lateral ectoblasts H1,H2, V1-V6 and T and cells in the gonad primordium (data not shown). These results show that neither the timing of S-phase nor the specific cells that enter S-phase are affected by the *ncc-1* mutations.

The complete genome is replicated in *ncc-1* mutants

The results described above indicate that *ncc-1* is strictly required for M phase and not for G_1 progression or DNA replication. However, we wished to examine two alternative explanations. First, it remained possible that cells in *ncc-1* mutants initiate DNA synthesis but fail during the elongation phase. Second, perdurance of maternal product could formally allow initiation of the first division cycle in the postembryonic lineages. To address these issues, we compared the amounts of DNA in intestinal nuclei of adult wild-type animals and *ncc-1* mutants. Wild-type animals hatch with 20 diploid intestinal cells. In general, the 14 most posterior of these undergo one round of DNA synthesis followed by nuclear division in late L1 stage (Hedgecock and White, 1985; Sulston and Horvitz, 1977 and Table 2). In addition, during each larval stage all intestinal nuclei undergo S-phase without mitosis, leading to a 32n DNA content by the adult stage.

We fixed adult wild-type and *ncc-1* animals and stained them with the DNA stain propidium iodide, and determined the DNA content of the intestinal cells by quantitative confocal analysis. Body wall muscles are

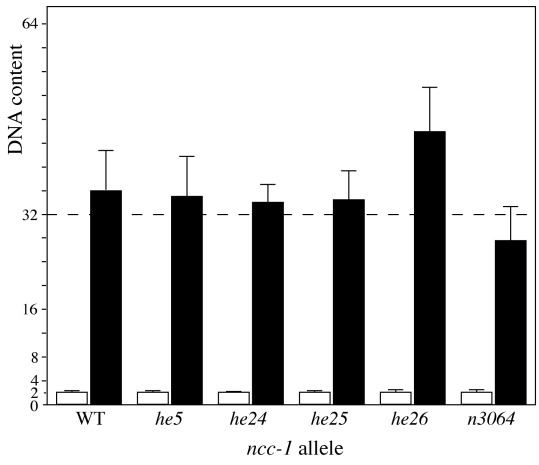

Fig. 7. Intestinal cells in wild-type and *ncc-1* animals are equally polyploid (black bars). Body muscle cells are used as an internal 2n standard (white bars). Each bar indicates the average DNA content ± s.d., of 10 nuclei in 2 independent animals.

diploid throughout postembryonic development in wild-type animals. Using body muscle nuclei as an internal 2n DNA standard, we found that the intestinal nuclei in *ncc-1* mutants and wild-type animals were equally polyploid and contained close to 32n amounts of DNA (Figs 6C, 7). The posterior nuclei did not have higher amounts of DNA than the anterior nuclei, suggesting that the lack of mitosis in *ncc-1* mutants prevents the second S phase that normally follows this division during L1 development. Together, these results show that cells in *ncc-1* mutants can replicate their entire genome even late in development. Thus, *ncc-1* appears to be required specifically for M phase.

DISCUSSION

The family of cdc2-related kinases in *C. elegans*

In this study, we have identified members of the family of cdc2-related kinases in *C. elegans*. In vertebrates, the kinases that are most closely related to *cdc2*, can be divided into 4 different groups (Meyerson *et al.*, 1992), and a member of each of these subfamilies appears to be present in *C. elegans*.

The most extensively studied subfamily consists of CDK1/Cdc2, CDK2 and CDK3. These kinases are closely related in sequence, and each of the human kinases can complement *CDC28* mutations in *S. cerevisiae*. CDK2 and CDK1/Cdc2 are activated sequentially in the cell cycle and studies in a number of systems indicate distinct functions. Immunodepletion of the *Xenopus* homologs showed a specific requirement for CDK2/Eg1 during S-phase and for Cdc2 during M phase (Fang and Newport, 1991). Antibody-injection studies and expression of dominant-negative mutants implicated CDK2 and CDK3 in G_1-S control and CDK1 in G_2-M regulation in mammalian cells (Pagano *et al.*, 1993; Riabowol *et al.*, 1989; Tsai *et al.*, 1993a; van den Heuvel and Harlow, 1993). Finally, a CDK1 mutation has been identified as the genetic lesion in a mouse cell line with a temperature-sensitive G_2 arrest phenotype (Th'ng *et al.*, 1990). The *C. elegans* kinase *ncc-1* belongs in this subfamily; it shares more than 60% identity with the three human kinases in this group and can complement the *CDC28-1N* mutation in *S. cerevisiae* (Mori *et al.*, 1994). Our studies further define NCC-1 as the ortholog of CDK1/Cdc2. At present, no homologs of CDK2 or CDK3 have been identified, although K03E5.3 and B0285.1 are predicted to encode candidates that are 42% and 41% identical to CDK2 (see below).

Human CDK4 and CDK6 form a subgroup of two kinases that share 71% amino acid identity among each other. These kinases are more distantly related to the CDK1/2/3 subfamily and they have not been observed to complement *CDC28* mutations in yeast (Meyerson *et al.*, 1992). Based on their association with D-type cyclins and timing of activation, CDK4 and CDK6 have been implicated in G_1 control. Moreover,

the kinase inhibitor p16 can bind to and inactivate CDK4 and CDK6 and overexpression of p16 can cause G_1 arrest in human cells (Sherr, 1996; Sherr and Roberts, 1995). Results from studies in mammals suggest a pathway for G_1 control in which the tumor suppressor p16 acts upstream of the oncogene products CDK4, CDK6 and Cyclin D, which negatively regulate the retinoblastoma protein, pRB (Sherr, 1996). At present, neither CDK4 nor CDK6 have been shown directly to have essential cell-cycle functions. In fact, deletion of the *Drosophila Cdk4/6* gene was found to cause reduced fertility but not a general cell-cycle arrest (C. Lehner, personal communication). The product of H06A10.1 is most related to this subgroup and is predicted to share 37% identity with human CDK4 (33% with CDK6). In RNAi experiments with this kinase, we and others have only identified very limited defects in cell division (M. Park and M. Krause, personal communication).

A third subfamily consists of three closely related PCTAIRE kinases in humans. *C. elegans* PCT-1 is ~80% identical to any one of the three human PCTAIRE kinases within a 240 amino-acid fragment of the catalytic core. Cyclin association has not been described for these kinases. In fact, PCTAIRE kinases contain N-terminal and C-terminal extensions that could act as regulators in cis. Such N- and C-terminal extensions are also present in the *C. elegans* PCT-1 kinase, and measure 234 and 48 amino acids respectively. PCTAIRE message and protein are abundantly expressed in human and rodent brains (Hirose *et al.*, 1997; Le Bouffant *et al.*, 1998; Meyerson *et al.*, 1992). By developmental northern blotting, we detected *pct-1* message during all developmental stages in the worm (data not shown). The *pct-1* mRNA levels were not found to correlate with mitotic divisions but were strongly elevated in young adults. We did not detect any abnormalities in *pct-1* dsRNA-injected animals or their progeny.

A single kinase, CDK5, that is highly conserved in metazoans defines a distinct subfamily. CDK5 is closely related to the CDK1/2/3 kinases and yet no cell-cycle related functions have been identified. By contrast, CDK5 kinase activity can be detected specifically in post-mitotic neuronal cells (Tsai *et al.*, 1993b). CDK5 has been implicated in neurite outgrowth during neuronal differentiation (Nikolic *et al.*, 1996), and mice lacking CDK5 or its activator p35 display severe defects in cortical lamination (Chae *et al.*, 1997; Gilmore *et al.*, 1998). We did not observe obvious defects in neural functions in *cdk-5(RNAi)* animals.

Implications from RNAi studies

For a remarkable number of genes RNA interference has now been shown to cause strong loss-of-function phenotypes (e.g: Fire *et al.*, 1998; Tabara *et al.*, 1998). However, absence of an RNAi phenotype is little informative, especially because the mechanism of interference is not understood. Two aspects of RNA interference have been particularly useful in this study: RNAi offers the possibility to examine the function

of maternally contributed product and RNAi phenotypes can be used to direct the isolation of mutant alleles in genetic screens.

The first aspect relates to a common problem in studying genes that are required in early as well as later development. A single wild-type copy from the mother generally appears sufficient to provide maternal function and, despite early gene requirements, loss-of-function phenotypes may first become detectable during late embryogenesis or early larval development (Ahnn and Fire, 1994; Storfer-Glazer and Wood, 1994). However, RNA interference is particularly efficient in blocking maternal contribution, and RNAi allowed us to examine *ncc-1* gene function during early embryogenesis. Following RNAi, NCC-1 protein levels were reduced below the detectable level even in the germline of injected animals. This way, we were able to detect requirement for *ncc-1* in mitotic and meiotic division in the germline.

Another application of RNAi is first demonstrated in this study: we used RNAi to predict the *ncc-1* loss-of-function phenotype and subsequently identified *ncc-1* mutations in animals with similar phenotypes. In an F2 screen, homozygous mutants are likely derived from mothers with one functional gene copy and hence display zygotic phenotypes. For three genes that are both maternally and zygotically required, *lin-5*, *lin-6* and *ncc-1*, we have observed that some of the early brood after RNA injection closely mimic zygotic phenotypes. Such RNAi larval phenotypes may provide a powerful reverse-genetics approach for many other genes in *C. elegans*.

***ncc-1* acts to promote entry into mitosis**

NCC-1 is closely related to yeast CDC28/cdc2 as well as CDK1/Cdc2, CDK2 and CDK3 kinases in vertebrates. A second kinase with such a high degree of homology was not detected. Does *ncc-1* perform a role similar to *CDC28/cdc2*$^{+}$, regulating multiple transitions in the cell cycle, or does it act to regulate progression through a specific phase of the cell cycle? The data described here show that *ncc-1* is required specifically for meiotic and mitotic M phase, indicating that *ncc-1* is the ortholog of CDK1/Cdc2 in other metazoans.

The strongest support for this conclusion stems from the cell-cycle arrest caused by *ncc-1* mutations. Several lines of evidence indicate that four of the *ncc-1* mutations create strong or complete loss of function. Two of the alleles should produce truncated products that lack most of the conserved kinase domains: *he5* contains a deletion and nonsense mutation and *he26* contains a nonsense mutation at codon 68. In addition, the missense mutation identified in *n3064* causes a Glu-to-Lys substitution of one of nine residues that are nearly invariant in protein kinases. Based on crystal structure studies, this glutamate residue is expected to contact an equally conserved and oppositely charged Arg in the C-terminal end of the catalytic core (De Bondt *et al.*, 1993; Knighton *et al.*, 1991). The missense mutation in *he24* results in a Glu-to-Lys

change next to the PSTAIRE motif. The corresponding Glu 57 in human CDK2 is believed to form a hydrogen bond with Tyr 185 in cyclin A (Jeffrey *et al.*, 1995). Binding of cyclin A to the PSTAIRE domain probably contributes to kinase activation by changing both the orientation of ATP and the position of a kinase domain that blocks the catalytic cleft. The *he25* allele contains a missense mutation that affects a glutamate residue that is conserved in CDK1/Cdc2 and CDK2 but not in kinases outside the Cdc2 family. The phenotype caused by the *he25* mutation is partly temperature sensitive, indicating that this mutation does not create a null allele.

The *ncc-1* mutants developed normally through embryogenesis and failed all cell divisions after hatching. The first postembryonic cell cycles in *ncc-1* mutants appear to be normal in timing of G_1 and S phase, based on rnr::GFP expression and BrdU incorporation. However, chromosome condensation and nuclear envelope degradation do not occur. Thus, the cells likely arrest in G_2 and *ncc-1* appears required to promote progression into mitosis. This function is consistent with the role of CDK1/Cdc2 kinases in other metazoans. One minor difference exists with *Drosophila*, in which inactivation of the mitotic kinase activity has been observed to result in polyploidy (Hayashi, 1996). This difference may reflect the fact that many cells in *Drosophila* undergo endoreduplication after final division.

We expect that *ncc-1* is essential for all embryonic and postembryonic divisions. This is based on the observed *ncc-1* requirement for cell divisions in all postembryonic lineages and the fact that homologous CDKs are required for all mitotic divisions in other species. As explained above, maternal *ncc-1* product likely is sufficient for embryogenesis. However, this assumption lacks formal proof, as *ncc-1(RNAi)* embryos arrest prior to the zygotic division.

***ncc-1* is required for meiosis I**

Following *ncc-1* RNAi, fertilized oocytes fail to complete meiotic maturation. At the time of injection, a large number of germ precursor cells are in pachytene of meiosis I. These nuclei progress through meiotic prophase I but do not initiate metaphase, indicating that *ncc-1* is required to promote the transition from prophase to metaphase in meiosis. A role in meiosis is consistent with the function of CDK1/Cdc2 in other eukaryotes. In fact, *Xenopus* $p34^{cdc2}$ was discovered by the biochemical characterization of maturation promoting factor (MPF), a cytoplasmic factor that induces meiotic maturation when injected into immature oocytes (Masui and Markert, 1971). Completion of meiosis I and II is a two step process in amphibians. Progesterone, or injection of MPF, triggers oocytes that are arrested in diplotene of prophase I to progress through meiosis I. Mature oocytes will subsequently arrest in metaphase of meiosis II and can be triggered by fertilization to complete meiosis (Masui and Markert, 1971). Meiotic maturation in *C. ele-*

gans is induced by a factor in sperm that is independent from the sperm's function at fertilization (McCarter *et al.*, 1999). In the absence of sperm, *C. elegans* oocytes arrest for prolonged periods in diakinesis (McCarter *et al.*, 1999). Future studies may reveal whether progesterone and the 'sperm factor' activate similar pathways that induce $p34^{cdc2}$ and *ncc-1*, respectively, and trigger progression to meiotic metaphase I.

Does progression through meiotic prophase require *ncc-1* function? In the RNA-injected animals, oocyte development appears normal and chromosomes become fully condensed. We cannot exclude that inactivation of *ncc-1* by RNAi was incomplete. However, several observations support effective inactivation. First, upon injection of *ncc-1* dsRNA, NCC-1 protein levels were reduced below the level of detection (Fig. 2G,I). Second, *ncc-1* RNAi interfered with cell division in the mitotic part of the germ-line and neutralized phosphorylation of histone H3 in mitotic germ cells as well as oocytes. Thus, a potent effect from RNAi was detected throughout the entire gonad. Finally, injection of dsRNA at a concentration of 0.01 mg/ml was sufficient to completely arrest meiotic maturation, whereas even a 100-fold higher concentration did not affect progression through prophase of meiosis I. If inactivation was complete, entry into meiotic development, meiotic prophase progression, condensation of chromosomes to diakinesis bivalents and oocyte development can all occur independent of *ncc-1*. If these processes do require *ncc-1* activity, this must be less activity than required for germ cell proliferation and for the transition from diakinesis to meiosis I. As yet, formation of diakinesis bivalents of normal morphology in the absence of CDK1 activity has not been described in any eukaryote. During spermatogenesis in *Drosophila*, DNA condensation can occur in the apparent absence of *Dmcdc2* activity (Sigrist *et al.*, 1995). However, male meiosis in *Drosophila* does not involve meiotic recombination and attachment of bivalents by chiasmata.

Nuclear envelope breakdown occurs slowly in mature *ncc-1(RNAi)* oocytes; by the time it is accomplished meiosis would normally have been completed. The fact that degradation still occurs could indicate incomplete inactivation of *ncc-1*, as CDK1 is believed to phosphorylate nuclear lamins and to trigger nuclear envelope breakdown in mitosis (Peter *et al.*, 1990). However, other kinases have also been implicated in nuclear lamin phosphorylation, including S6 kinase II (Ward and Kirschner, 1990) and protein kinase C (Hocevar *et al.*, 1993). Such kinases may trigger nuclear enveloppe breakdown in the absence of *ncc-1* activity.

How is progression through G_1/S regulated in *C. elegans*?

Our results provide an interesting paradox: *ncc-1* appears specifically required for the regulation of M phase, yet essential regulators of the G_1-S transition have not been identified. Such regulators are especially important as regulation of cell division by developmental signals in most

cells occurs during G_1 phase. Ectopic expression of the CDK inhibitor *cki-1* causes cell division arrest prior to S phase in *C. elegans* (Hong *et al.*, 1998), which is consistent with the assumption that G_1-S progression is dependent on CDK activity. In other metazoans, CDK2 appears to promote G_1/S transition, although genetic null phenotypes have not been described as yet. As the *C. elegans* genome has been completely sequenced, the *C. elegans* kinase that is most similar to CDK2 is likely encoded by the K03E5.3 gene. RNAi for this gene caused severe but highly variable developmental and cell division defects. More severe defects where seen when the same RNA was injected into heterozygous *ncc-1*/+ animals (data not shown). We are currently exploring whether K03E5.3 and *ncc-1* can act redundantly in G_1/S progression.

MATERIALS AND METHODS

Culture conditions and strains

Worm strains used were derived from the wild-type Bristol strain N2 and Bergerac strain RW7000. Worms were cultured using standard techniques as described by Brenner (Brenner, 1974). We used the following mutations, descriptions of which can be found in Riddle *et al.* (Riddle *et al.*, 1997) or cited references:

LGIII: *dpy-17(e164)*, *unc-36(e251)*, *dpy-19(e1259)*, *unc-32(e189)*, glp-1(q158), ncc-1(*he5*, he6, *he24*, *he25* and *n3064*) (this paper), *unc-47(e307)*, *unc-49(e382)*, *tra-1(e1099)* and *dpy-18(e364)*. Rearrangements: *qDp3(III,f)* (Austin and Kimble, 1987), *ctDp6(III,f)* (Hunter and Wood, 1990), *nDf40* (Graham and Kimble, 1993), *qC1 dpy- 19(e1259) glp-1(q339)* (Edgley *et al.*, 1995). Expression of an rnr::GFP reporter was examined in strains VT774 *unc-36(e251)*; *maIs103[rnr::GFP unc-36(+)]* (Hong *et al.*, 1998) and SV61 *ncc-1(n3064)/dpy-19(e1259) unc-47(e307); maIs103[rnr::GFP unc-36(+)]*.

Cloning of cdc2-related genes

To clone *cdc2*-related genes from *C. elegans*, we used RT-PCR amplification with degenerate oligonucleotide primers, low stringency hybridization of a cDNA library and antibody screening of an expression library. For the reverse transcriptase PCR approach, RNA was isolated from mixed stage *C. elegans* cultures grown in bulk (Wood, 1988). First strand cDNA was prepared from total RNA and amplified by PCR, using degenerate oligonucleotides derived from the highly conserved motifs EKIGEGTY and EGVPSTA as 5' primers and DLKPQNL and WYRSPEV as 3' primers (primer sequences available upon request). PCR fragments were cloned into pBluescript II SK (Stratagene) and sequenced by the dideoxy method. Partial clones were obtained this way from *ncc-1*, *cdk-5* and *pct-1*. To obtain full length cDNAs, a nematode cDNA library cloned in the Uni-ZAP vector (Stratagene) was probed with PCR fragments labeled with [α-32P]dCTP. Although low stringency conditions were used, we obtained only cDNA clones from *ncc-1*, *cdk-5* and *pct-1*. The same library was induced with IPTG and probed with a mouse monoclonal antibody recognizing the PSTAIRE motif (a kind gift from M. Yamashita). This resulted in identification of multiple cDNAs that were all derived from the *ncc-1* gene.

We expect the predicted kinases to be full length based on the isolation of multiple cDNAs of similar size, homology in the N terminus with the human kinases and a nonsense codon preceding the ATG initiation codon in the *pct-1* gene. The genomic localization was identified for each gene by probing YAC contigs, followed by PCR analysis of cosmids from the region. Cosmids T05G5, C07G1 and R11C7 were found to contain the *ncc-1*, *pct-1* and *cdk-5* genes, respectively, and were used to determine the gene structures

by Southern blotting and DNA sequencing. Subsequently, all sequences have been confirmed by the *C. elegans* sequencing project.

RNA interference

Plasmids containing full length *ncc-1*, *cdk-5* and *pct-1* cDNAs were used for *in vitro* transcription. For B0285.1, a genomic fragment was amplified by PCR using primers 5'-agaattcacaatgcttgatcaaattggc and 5'-aaggatccagattgtttttctttttccg. For, K03E5.3 a genomic fragment was amplified using primers 5'-aatccgcaattttgatgactccag and 5'-caaccgatatcgctccaggtaagc. These fragments were cloned into pBluescript SK. A plasmid containing cDNA yk492e2, derived from H06A10.1, was kindly provided by M. Park, M. Krause and Y. Kohara. Templates were linearized and transcribed *in vitro* with T3 or T7 RNA polymerase. RNA was phenol extracted, ethanol precipitated and dissolved in TE (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA) to a final concentration of 1 mg/ml. A 1:1 mixture of the two strands was injected into young adult N2 hermaphrodites as described (Fire *et al.*, 1998).

Screening for *ncc-1* alleles

N2 animals were mutagenized with 50 mM ethylmethanesulfonate (EMS) as described by Brenner (Brenner, 1974). Mutagenized L4 hermaphrodites were picked to 100 mm plates, 30 per plate, and allowed to produce self progeny at 25°C. F1 animals in the fourth larval stage were picked to 60 mm plates, four per plate, and their progeny were examined for mutants that resemble larval-arrested *ncc-1(RNAi)* animals. Mutants that were sterile, uncoordinated, slightly dumpy and had relatively large head regions were examined by Nomarski microscopy to determine defects in cell division. Candidate mutations were recovered from siblings and were mapped initially by PCR, making use of primers based on polymorphic Sequence-Tagged Sites in the RW7000 Bergerac strain (Williams *et al.*, 1992). *ncc-1* mutations mapped to chromosome III and were placed by standard three factor crosses (Wood, 1988) between *dpy-19(e1259)* and *unc-47(e307)* or *dpy-17(e164)* and *tra-1(e1099)*, about 1.06 map units to the right of *dpy-19* at position 0.89. In addition, all mutations failed to complement *nDf40* and allele *n3064*.

Antibodies

Antibodies were used in the following dilutions: mouse monoclonal antibody DM1A against α-tubulin (Sigma) diluted 1:1000 for western blot analysis and 1:500 for immunostaining, rabbit polyclonal antibody against phosphorylated histone H3 (Upstate Biotechnology) diluted 1:400, mouse monoclonal antibody BU-33 against BrdU (Sigma) diluted 1:60, mouse monoclonal antibody against the PSTAIRE region of Cdc2 (a kind gift from M. Yamashita), used 1:1000 diluted for immunoblotting and 1:250 diluted for immunostaining. A polyclonal antiserum raised against the C terminus of NCC-1 was diluted 1:1000 for immunostaining (a kind gift from J. Schumacher and A. Golden). Donkey-anti-mouse and donkey-anti-rabbit IgG secondary antibodies, rhodamine- or FITC-conjugated, were from Jackson labs and diluted 1:200.

Western blot analysis and Immunostaining

To obtain protein samples, synchronized wild-type animals were washed 4 times in M9 buffer and dissolved by boiling for 5 minutes in 1x Laemmli protein loading buffer (Harlow and Lane, 1988). Protein samples were separated by SDS-PAGE followed by coomassie staining, to determine protein concentration, or immunoblotted using standard procedures (Harlow and Lane, 1988).

For antibody staining of germlines and embryos, gravid wild-type hermaphrodites were dissected in a drop of M9 buffer on a gelatin-coated slide. A coverslip was applied and the slide frozen on dry ice. After 10 minutes the coverslip was cracked off and slides were incubated in methanol at -20°C for 20 minutes and acetone at -20°C for 20 minutes. For immunostaining of larvae, animals were fixed in Bouin's solution and antibody staining was performed according to standard procedure (Harlow and Lane, 1988; Nonet *et al.*, 1997). For staining of DNA only, animals were fixed overnight in Carnoys solution

(60% ethanol, 30% glacial acetic acid, 10% chloroform) and slowly rehydrated in PBS + 0.1% Tween-20. In all procedures, DNA was stained with either propidium iodide (PI) 1 mg/ml, following treatment with 200 μg/ml RNAse A for 30 minutes at 37°C, or with 1mg/ml 4,6-diamidino-2-phenylindole (DAPI) (Sigma). Samples were mounted on slides in 10% PBS, 2.3% Dabco, glycerol.

Images were obtained using a Sensys cooled CCD camera (Photometrics, Tucson, AZ) and a Power Macintosh computer. Image analysis and computational deconvolution was performed with Openlabs software version 1.7.8 (Improvision, Boston, MA). Images were pseudocolored and merged using Adobe Photoshop.

BrdU labeling

Synchronized cultures were obtained from embryos isolated by hypochlorite treatment and hatched in the absence of food (Wood, 1988). The resulting early L1 arrested larvae were transferred to plates with bacteria, which triggers highly synchronized development. DNA synthesis was visualized by incorporation of BrdU. A solution of BrdU in S-medium was added to plates with synchronized larvae to a final concentration of 0.5 mg/ml. Animals were fixed in Bouin's fixative and stained with anti-BrdU antibodies. The staining procedure was as described above with one addition; prior to preadsorbtion, DNA was denatured by incubating the fixed animals for 2 hours at room temperature in a 1:1 mixture of 4 M HCl and PBS + 1% BSA + 0.5% Triton X-100.

DNA quantitation

To quantitate DNA content, series of 32 Z-sections were taken of propidium iodide-stained animals with a confocal scanning laser microscope (Leica). Total pixel intensities were calculated for each section on a Macintosh computer using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/). Pixel intensities of all 32 sections were added and recalculated to DNA content, using body wall muscle nuclei as a 2n DNA standard.

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Chapter 3

lin-35 Rb and *cki-1* Cip/Kip cooperate in developmental regulation of G_1 progression in *C. elegans*

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ABSTRACT

We have investigated the regulation of cell-cycle entry in *C. elegans*, taking advantage of its largely invariant and completely described pattern of somatic cell divisions. In a genetic screen, we identified mutations in *cyd-1* Cyclin D and *cdk-4* CDK4/6. Recent results indicated that during *Drosophila* development, Cyclin D-dependent kinases regulate cell growth rather than cell division. However, our data indicate that *C. elegans cyd-1* primarily controls G_1 progression. To investigate whether *cyd-1* and *cdk-4* solely act to overcome G_1 inhibition by retinoblastoma family members, we constructed double mutants that completely eliminate the function of the retinoblastoma family and Cyclin D-CDK4/6 kinases. Inactivation of *lin-35* Rb, the single Rb-related gene in *C. elegans*, substantially reduced the DNA replication and cell-division defects in *cyd-1* and *cdk-4* mutant animals. These results demonstrate that *lin-35* Rb is an important negative regulator of G_1/S progression and probably a downstream target for *cyd-1* and *cdk-4*. However, as the suppression by *lin-35* Rb is not complete, *cyd-1* and *cdk-4* probably have additional targets. An additional level of control over G_1 progression is provided by Cip/Kip kinase inhibitors. We demonstrate that *lin-35* Rb and *cki-1* Cip/Kip contribute nonoverlapping levels of G_1/S inhibition in *C. elegans*. Surprisingly, loss of *cki-1*, but not *lin-35*, results in precocious entry into S phase. We suggest that a rate limiting role for *cki-1* Cip/Kip rather than *lin-35* Rb explains the lack of cell-cycle phenotype of *lin-35* mutant animals.

INTRODUCTION

The development of a multicellular organism requires the careful coordination of cell division with growth and differentiation. In part, this coordination is achieved through integration of extracellular signals during the G_1 phase, to which cells respond by either advancing into or withdrawing from another division cycle (Pardee, 1989). Ultimately, mitogenic and antiproliferative signals affect the cell-intrinsic cell-cycle machinery, of which the cyclin-dependent kinases (CDKs) are key components. The importance of the G_1 control mechanisms is underscored by the finding that most, if not all, tumor cells have defects in one or more genes involved in G_1 progression (Sherr, 1996). Despite extensive research, the *in vivo* functions of such genes have been poorly characterized.

Significant insights have been gained from studying mammalian cells in tissue culture and gene alterations in human cancer. Members of the retinoblastoma (Rb) tumor suppressor family (pRb, p107 and p130 in mammals) have been found to inhibit progression through the

G_1 phase (Sherr, 1996). This negative-regulatory function of the pRb protein is constrained by phosphorylation at multiple CDK phosphorylation sites. Sequential phosphorylations disrupt the binding between pRb and transcription factors such as E2F, thereby allowing these transcription factors to activate genes required for DNA synthesis and removing active transcriptional repression by pRb (Dyson, 1998). One of the critical targets of the E2F transcription factor is Cyclin E (Dyson, 1998), which together with its partner CDK2 is required for the initiation of DNA replication (Duronio and O'Farrell, 1995; Knoblich *et al.*, 1994; Ohtsubo *et al.*, 1995; Tsai *et al.*, 1993; van den Heuvel and Harlow, 1993).

CDKs appear to act at multiple levels of this G_1 control pathway. The first CDKs to become active in the cell cycle consist of a CDK4 or CDK6 catalytic subunit and a D-type cyclin regulatory subunit (Sherr, 1993). It is generally thought that Cyclin D-dependent kinases initiate pRb phosphorylation in mid G_1, while the subsequent activation of Cyclin E-CDK2 kinases leads to completion of this process (Mittnacht, 1998). However, the presence of multiple D-type cyclins, CDK4/6 kinases and pRb-related proteins has hampered a direct demonstration of their functions *in vivo*. For example, it is not clear to what extent Cyclin D-CDK4/6 kinases are essential for pRb inactivation and cell-cycle progression *in vivo*. Moreover, it remains unknown whether Cyclin D has critical targets other than proteins of the pRb family. Potential targets include cyclin-dependent kinase inhibitors (CKIs) of the Cip/Kip family (Sherr and Roberts, 1999). $p21^{CIP1}$ and $p27^{KIP1}$ associate with Cyclin E-CDK2 complexes and prevent their kinase activity. The same inhibitors also bind Cyclin D and CDK4/6, but apparently mediate their assembly rather than inhibiting kinase activity (Cheng *et al.*, 1999; LaBaer *et al.*, 1997). This association may result in sequestering the Cip/Kip inhibitors from Cyclin E-CDK2 complexes, which further promotes progression through the G_1/S transition.

Studies in genetic model systems should contribute further insights in the *in vivo* functions of G_1 regulatory genes. Such analyses have already provided surprising results. For example, recent studies in *Drosophila* revealed a requirement for CDK4 in cell growth, rather than cell division (Datar *et al.*, 2000; Meyer *et al.*, 2000). The nematode *C. elegans* provides an attractive animal model for cell-cycle studies. The somatic cell-lineage of *C. elegans* is largely invariant and has been completely described; thus, the timing of division is known for every cell (Sulston and Horvitz, 1977; Sulston *et al.*, 1983). In addition, most of the cell-cycle regulators mentioned above are represented by single genes in *C. elegans*, which simplifies their functional analysis. For example, a single D-type cyclin (*cyd-1*), CDK4/6-related kinase (*cdk-4*), Cyclin E (*cye-1*) and pRb family member (*lin-35* Rb) are present (Fay and Han, 2000; Lu and Horvitz, 1998; Park and Krause, 1999; The *C. elegans* Sequencing Consortium, 1998). In addition, two genes, *cki-1* and *cki-2*,

encode cyclin-dependent kinase inhibitors of the Cip/Kip family (Feng *et al.*, 1999; Hong *et al.*, 1998; The *C. elegans* Sequencing Consortium, 1998). Inhibition of gene function by RNA interference has revealed roles for *cyd-1/cdk-4* and *cki-1* as positive and negative regulators of the G_1/S transition, respectively (Hong *et al.*, 1998; Park and Krause, 1999). However, the network of gene activities that regulates G_1 progression in *C. elegans* remains entirely unknown. In fact, *lin-35* Rb was identified as a member of the 'synthetic Multivulva' (synMuv) genes that inhibit the expression of vulval cell fates (Ferguson and Horvitz, 1989), and a function in cell-cycle regulation has not been reported for *lin-35* Rb.

To identify G_1 regulators in *C. elegans*, we performed a screen for mutants that arrest cell-division in G_1 phase. In this screen we isolated mutations in *cyd-1* Cyclin D and *cdk-4* CDK4/6. We used these alleles to address the interaction between Cyclin D-dependent kinases and pRb family members, by creating double mutant animals that lack activity of *cyd-1/cdk-4* and *lin-35*. Our results demonstrate that *lin-35* Rb is an important negative regulator of cell division and probably a major downstream target of *cyd-1/ cdk-4*. However, *lin-35* Rb did not appear to be the only target of *cyd-1/ cdk-4*. In addition, we provide evidence that the Cip/Kip family members *cki-1* and *cki-2* cooperate with *lin-35* Rb in controlling cell-cycle entry, and that these two pathways provide non-overlapping levels of cell-cycle control.

RESULTS

Identification of a D-type cyclin and CDK4/6 related kinase in a screen for positive regulators of G_1 progression

To identify positive regulators of G_1 progression, we used a reporter construct with S phase-specific transcription; the green fluorescent protein (GFP) expressed under the control of *ribonucleotide reductase* promoter sequences (*rnr::GFP,* Hong *et al.*, 1998). The F2 progeny from mutagenized animals carrying the *rnr::GFP* transgene were examined for the presence of mutants that lack postembryonic cell division and expression of the *rnr::GFP* marker (Fig.1A,B). We identified six independent mutations that fulfill these criteria. Initial mapping placed two mutations (*he112* and*he116*) on chromosome II and four mutations (*he109*, *he110*, *he111* and *heDf1*) on the X chromosome.

he112 and *he116* failed to complement each other, indicating they may affect the same locus. Standard three-factor mapping placed both mutations in the proximity of the cell-cycle regulatory gene *cyd-1*, which encodes the sole D-type cyclin in *C. elegans* (Park and Krause, 1999). Molecular lesions that affect the *cyd-1* gene were identified by DNA sequence analysis (*he112*) and by PCR and Southern blotting experiments (*he116*). *cyd-1*(*he112)* contains a nonsense mutation predicted to truncate the C-terminal 114 amino acids (Fig. 2). The *cyd-1(he116)* muta-

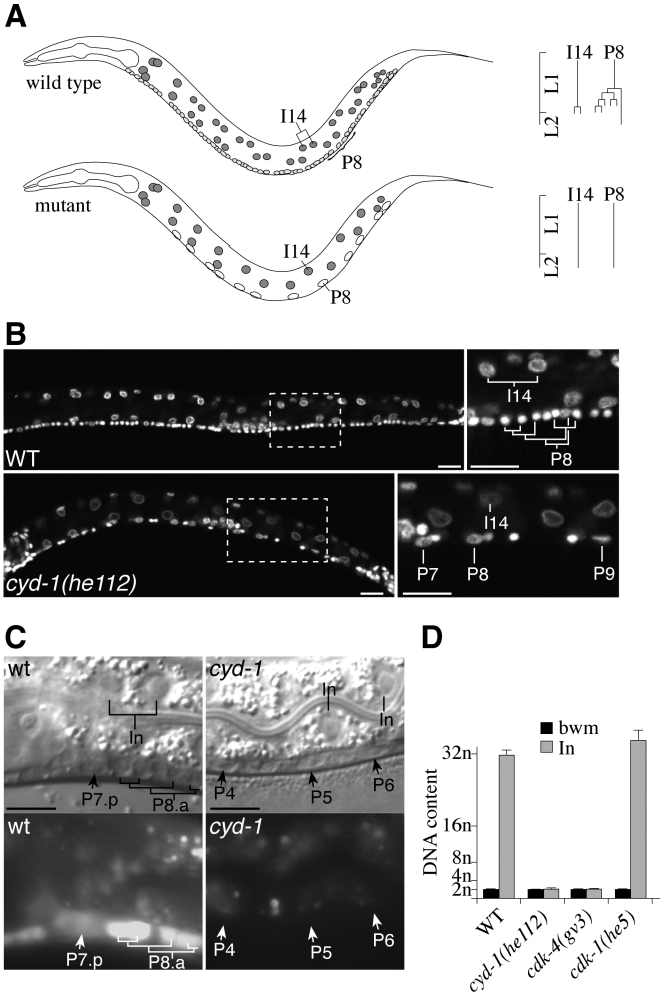

Fig. 1. Identification and characterization of *cyd-1* and *cdk-4* mutant animals. (A) Positions of cells in the ventral cord precursor (P) and intestinal (I) lineages in late L1 wild type (top) and cell-cycle mutant (bottom) larvae. The lineages of an individual P and I cell are indicated for each genotype (right). (B) Postembryonic blast cells remain undivided in *cyd-1* and *cdk-4* mutants, as indicated for intestinal and P precursor cells in the enlarged sections (right). The panels show a late L1 wild-type larva (top) and similar stage *cyd-1(he112)* mutant (bottom) after fixation and DNA staining with propidium iodide. (C) Expression of the *rnr::GFP* S-phase marker in wild-type animal (left) and *cyd-1(he112)* mutant (right). Nomarski images (top) and corresponding epifluorescent images (bottom) show several cells of the P and intestinal lineages. In the *cyd-1* animal, only autofluorescence of the intestinal cells is detectable. (D) Quantitative measurements of DNA content in the intestinal nuclei (gray bars) of wild-type animals and mutant strains of indicated genotype. Body wall muscle cells (black bars) serve as 2n DNA standards. Scale bars: 10 μm. Values indicated are mean±s.e.m.

tion deletes the *cyd-1* promoter region as well as the first two exons (Fig. 2).

The predicted partner for CYD-1 is CDK-4, a CDK4- and CDK6-related kinase encoded by the *cdk-4* gene located on the X-chromosome (Park and Krause, 1999). Introduction of a wild-type *cdk-4* transgene in germline transformation experiments completely suppressed the defects caused by three X-linked mutations, *he109*, *he110,* and *he111*. Sequence analysis identified point mutations within the *cdk-4*-codingsequences in each of the three mutant strains. Alleles *he109* and *he111* contain nonsense mutations that should terminate translation after 207 and 291 amino acids, respectively (Fig. 2). The *he110* allele contains a missense mutation that converts a glutamate at position 85, which is conserved in protein kinases, to lysine (Fig. 2).

The mutant phenotype associated with *heDf1*, the fourth X-linked mutation, was not rescued by a wild-type *cdk-4* transgene. However, this mutation did map in the proximity of *cdk-4*, and failed to complement the *cdk-4(gv3)* allele. PCR and Southern blotting experiments revealed that *heDf1* is a deletion that removes the entire *cdk-4* gene (Fig. 2). In addition to the cell-cycle arrest, *heDf1* mutant animals displayed defects in growth and morphology that were not observed in animals homozygous for any of the other *cdk-4* alleles, including animals homozygous for the *cdk-4(gv3)* deletion. Because of the lack of rescue and pleiotropic defects, we conclude that *heDf1* deletes *cdk-4* and probably another gene required for larval development. *heDf1* was not analyzed further.

All mutations isolated were recessive and conferred fully penetrant cell-cycle defects (Table 1). Moreover, the defects observed in both *cdk-4* and *cyd-1* mutants were at least as severe as those caused by RNA-mediated interference (RNAi) of these genes (Park and Krause, 1999). Based on these genetic and molecular characterizations, the *cyd-1* and *cdk-4* alleles confer strong loss-of-function or null phenotypes.

cyd-1 and *cdk-4* are required for G_1/S progression in postembryonic cell divisions

Following a reverse genetics approach, Park and Krause (Park and Krause, 1999) previously identified essential roles for *cyd-1* and *cdk-4* in cell division. We compared the *cyd-1* and *cdk-4* mutant phenotype to the reported defects caused by *cyd-1* RNAi and the *cdk-4(gv3)* deletion allele. Animals homozygous for the *cyd-1* alleles *he112* and *he116* or *cdk-4* alleles *he109, he110,* and *he111* completed embryogenesis. The only embryonic

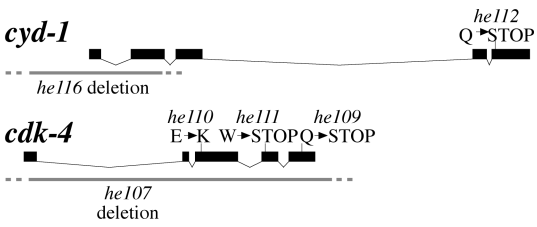

Fig. 2. Molecular lesions in *cyd-1* and *cdk-4* mutant alleles, illustrated with respect to the genomic structure. Exons are shown as boxes, introns as lines.

Table 1. Characterization of *cyd-1* and *cdk-4* alleles

Parental genotype	% larval arrest of progeny (n)*	Postembryonic blast cells that initiate division during the L1 stage	
		P2-P10	I
Wild type	0.0 (>1000)	9/9	14/20
cyd-1(he112)/mnC1	25.50 (697)	0/9	0/16
cyd-1(he116)/+	24.14 (994)	0/9	0/16
cdk-4(he109)/+	25.16 (930)	0/9	0/20
cdk-4(he110)/+	24.73 (1314)	0/9	0/20
cdk-4(he111)/+	23.65 (985)	0/9	0/20

*The percentage larval arrest was determined by counting the total progeny from three to five heterozygous animals of the indicated genotype.

Divisions of P and I cells were scored in L3 stage mutants fixed and stained with the DNA stain PI. Divisions of P1, P11 and P12 could not be unambiguously determined and, therefore, are excluded.

Cell divisions are represented as number of cells divided/number of cells present in individual animals.

More than 10 animals of each genotype were examined.

defect we observed was a failure of *cyd-1* mutants, but not *cdk-4* mutants, to complete the final few embryonic intestinal divisions. Consequently, *cyd-1(he112)* and *cyd-1(he116)* larvae hatched with 16 intestinal cells (16.0, n=10), rather than the 20 cells present in wild-type animals. Although wild-type maternal product could mask an embryonic role of *cyd-1* and *cdk-4* in homozygous mutants, RNAi of *cyd-1* or *cdk-4* also did not cause embryonic lethality and prevented cell division only during larval development (Park and Krause, 1999). As RNAi usually impedes both maternal and zygotic gene function, *cyd-1* and *cdk-4* appear to be predominantly required for postembryonic somatic cell cycles.

Two observations indicate that *cyd-1* and *cdk-4* mutant larvae arrest cell divisions prior to S phase. As first observed in the screen, each *cyd-1* or *cdk-4* mutant lacked detectable expression of the *rnr::GFP* S-phase reporter in the postembryonic lineages(Fig. 1C; Z1 and Z4, the somatic gonad precursor cells, were the only exception). In addition, we found no evidence of DNA replication in postembryonic cell lineages. Specifically, we determined the DNA content of cells in two postembryonic lineages: precursor cells of the ventral nerve cord (P), which undergo four rounds of cell division during the first larval stage, and intestinal nuclei, 14 of which divide once after hatching (Sulston and Horvitz, 1977). After this division, all intestinal nuclei go through a round of endoreduplication during each larval stage, resulting in a 32n DNA content (Hedgecock and White, 1985). The P and intestinal cells arrested with a 2n DNA content in both *cyd-1* and *cdk-4* mutants (Fig. 1D and not shown). By contrast, 4n and 32n DNA contents were found in the P and intestinal lineages, respectively, of *ncc-1/cdk-1* mutants whose cells arrest

after DNA synthesis in the G_2 phase (Boxem *et al.*, 1999, Fig. 1D). Expression of *ribonucleotide reductase* normally coincides with DNA replication, but does not depend on it (Duronio and O'Farrell, 1994). The fact that cells arrest with 2n DNA amounts and lack *rnr::GFP* expression shows that the cell-cycle arrest occurs before S phase.

Although our results largely correspond to those described by Park and Krause (Park and Krause, 1999), the *cyd-1* mutant phenotype was slightly more severe than the reported *cyd-1(RNAi)* phenotype. The *cyd-1(RNAi)* animals hatched with 20 intestinal cells that arrested with a 4n DNA content (Park and Krause, 1999), while *cyd-1(he112)* and *cyd-1(he116)* mutant larvae have 16 intestinal cells that arrest with a 2n DNA content. The simplest explanation for the difference is that RNAi did not completely inactivate *cyd-1* function. Our data further support the conclusion that *cyd-1* and *cdk-4* are essential for G_1/Sprogression in all postembryonic cell divisions.

cyd-1 and *cdk-4* are primarily required for cell division and not cell growth

An essential role for *cyd-1* and *cdk-4* in G_1 progression contrasts with results obtained in the fruit fly. *Drosophila cdk4* is not essential for most divisions; rather the Cdk4/Cyclin D complex has been implicated in regulation of cell growth (Datar *et al.*, 2000; Meyer *et al.*, 2000). To examine the possibility that the cell-division defects observed in *cyd-1* and *cdk-4* mutants are a secondary consequence of a cell-growth defect, we compared the growth rates of *cyd-1(he112)* and *cdk-4(gv3)* mutants with wild-type larvae. Until approximately 20 hours of larval growth at 15°C, *cyd-1(he112)* and *cdk-4(gv3)* mutants and wild-type siblings were indistinguishable in size (Fig. 3). After 20 hours, the growth rate of the wild-type siblings increased, whereas *cyd-1(he112)* and *cdk-4(gv3)*mutants continued to grow at a slow rate. The first larval (L1) stage ends with a molt at approximately 16 hours of postembryonic development at 15°C.

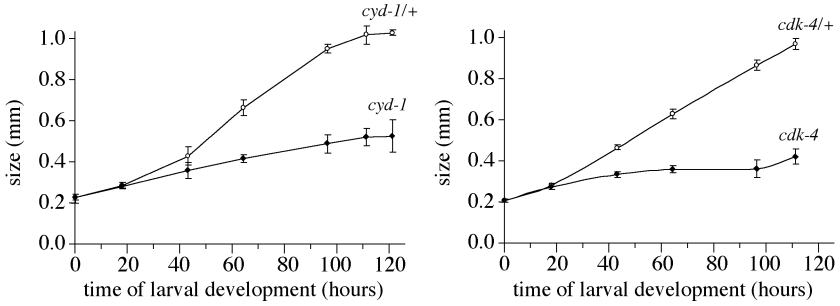

Fig. 3. *cyd-1* and *cdk-4* cell division defects precede growth defects. Size of *cyd-1(he112)* and *cdk-4(gv3)* homozygous mutants and wild type siblings (genotypes: *cyd-1(he112)/mnC1* and *cdk-4(gv3)/+* or *+/+)* is plotted as a function of time of postembryonic development at 15°C. The growth retardation of *cyd-1* and *cdk-4* mutants becomes first apparent in the L2 stage, subsequent to failure of some late embryonic and all postembryonic L1 divisions. Points indicate mean of five measured animals±s.d.

Cells in a variety of cell lineages divide during this stage in the wild-type, starting with the Q neuroblasts at approximately 5 hours of postembryonic development at 15°C. As none of these divisions occur in *cyd-1* and *cdk-4* mutants, the cell-division defects precede the growth-retardation phenotype. Moreover, the embryonic divisions take place in the absence of growth, yet the final intestinal divisions failed to occur during embryogenesis in *cyd-1* mutants. Together, our results indicate that *cdk-4* and *cyd-1* primarily promote cell-cycle entry in *C. elegans*, in agreement with the view derived from mammalian tissue culture experiments.

lin-35 Rb is an important negative regulator of G_1/S progression and a major downstream target of _cyd-1_ and _cdk-4_

Mammalian D-type cyclins in association with the CDK4 and CDK6 kinases phosphorylate members of the pRb protein family *in vitro* (Kato *et al.*, 1993; Meyerson and Harlow, 1994). However, it has been difficult to test whether this phosphorylation is crucial *in vivo* for inactivation of the G_1/S inhibitory function of the pRb protein, as multiple genes of each type (D-type cyclins, CDK4/6 kinases and Rb-family members) are present in mammals. By contrast, these different regulators are encoded by single genes in *C. elegans* (The *C. elegans* Sequencing Consortium, 1998), making this organism an ideal system in which to address whether Cyclin D and CDK4/6 are solely required to overcome G_1/S inhibition by pRb family members.

The single *Rb*-related gene in *C. elegans*, *lin-35*, was previously identified as a regulator of vulval cell-fate specification (Lu and Horvitz, 1998). Surprisingly, animals homozygous for *lin-35* presumed null mutations are viable and show no cell-division defects. If *cyd-1* and *cdk-4* only act to inhibit *lin-35* Rb, then the cell-cycle arrest of *cyd-1* and *cdk-4* mutants should be fully overcome by *lin-35* Rb inactivation. To test this hypothesis, we used several assays. First, we examined whether inactivation of *lin-35* in a *cyd-1* mutant background restores expression of the *rnr::GFP* S-phase marker. Mutations in *lin-35* had previously been shown to result in general suppression of transgene expression (Hsieh *et al.*, 1999). However, the *rnr::GFP* transgene was not silenced in the F1 progeny of animals injected with *lin-35* dsRNA (M. B. and S. v.d.H., unpublished). Importantly, inactivation of *lin-35* by RNAi efficiently restored *rnr::GFP* expression in *cyd-1* and *cdk-4* homozygous mutants (Fig. 4A).

Next we tested whether inactivation of *lin-35* could restore DNA replication in the intestinal nuclei. We constructed *lin-35;cyd-1* and *lin-35;cdk-4* double mutant strains, using two different alleles of *lin-35*, *n745* and *n2239*, which contain early nonsense mutations that probably completely eliminate *lin-35* function (Lu and Horvitz, 1998). Quantitative DNA measurements of intestinal nuclei showed that *lin-35(n745* or *n2239);cyd-1(he112)* and *lin-35(n745);cdk-4(gv3)* double mutant larvae were able to undergo multiple rounds of DNA replication, giving rise to a level of polyploidy in the double mutants that is similar to wild-type

animals (Fig. 4B). In addition to DNA replication, *lin-35;cyd-1* and *lin-35;cdk-4* double mutants reached near wild-type body and gonad size (not shown) and *lin-35(n745);cdk-4(gv3)* double mutants occasionally produced viable progeny. Thus, loss of function of *lin-35* Rb overcomes the G_1 arrest of cells in *cyd-1* and *cdk-4* mutants. These results demonstrate that *lin-35* Rb is an important inhibitor of the G_1/S transition and, by analogy with other systems, probably a major target of *cdk-4* and *cyd-1*.

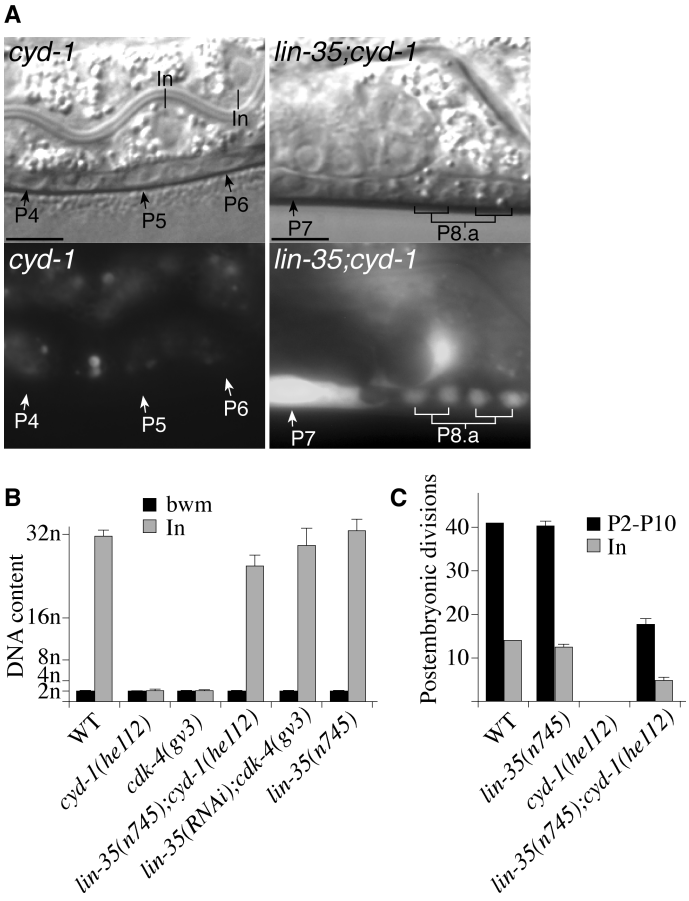

Fig. 4. *lin-35* acts as a negative regulator of cell-cycle progression. (A) Expression of the *rnr::GFP* S-phase marker in *cyd-1(he112)* (left) and *lin-35(RNAi); cyd-1(he112)* (right). Nomarski images (top) and corresponding epifluorescent images (bottom) show several cells of the P and intestinal lineages. In the *cyd-1* animal, only autofluorescence of the intestinal cells is detectable, whereas *lin-35* RNAi resulted in expression of *rnr::GFP* in the P cells and other lineages. (B) Quantitative measurements of DNA content in the intestinal nuclei (gray bars) of wild-type (WT) and mutant animals of indicated genotype. Body wall muscles (black bars) serve as 2n DNA standard. (C) Rescue of postembryonic cell divisions by *lin-35*. The cell number in the P2-P10 and intestinal lineages were counted in animals of indicated genetic backgrounds. Scale bars: 10 μm. Values indicated are mean±s.e.m.

The *C. elegans* genome contains single members of the D and E subfamilies of G_1 cyclins. Inactivation of *cye-1* Cyclin E by RNAi causes embryonic arrest at approximately the 100-cell stage (Fay and Han, 2000). Homozygous *cye-1* mutant larvae derived from heterozygous mothers display late larval defects and complete sterility (Fay and Han, 2000). RNAi for *lin-*35 did not affect the *cye-1(eh10)* mutant phenotype. Similarly, the embryonic arrest caused by *cye-1* RNAi was equally severe in a wild-type or *lin-35(n745)* mutant background (data not shown). Thus, *lin-35* inactivation specifically suppresses *cyd-1* and not *cye-1* loss of function. Although other interpretations are possible, these results are consistent with a model in which *lin-35* Rb acts downstream of *cyd-1/ cdk-4* and upstream of *cye-1*.

lin-35* is probably not the only target of *cyd-1* and *cdk-4

Although we observed substantial rescue of DNA replication, the number of cell divisions in the postembryonic lineages was not restored to wild-type levels in *lin-35;cyd-1* or *lin-35;cdk-4* double mutants (Fig. 4C). The double mutant animals also remained largely sterile. It appears unlikely that this lack of complete rescue resulted from incomplete inactivation of *lin-35* Rb, as probable null mutations were introduced (alleles *n745* and *n2239*) (Lu and Horvitz, 1998), and identical effects were observed following *lin-35* RNAi. Incomplete rescue is also unlikely to be caused by inactivation of a positive cell-cycle function of *lin-35* Rb, as *lin-35* single mutants do not display apparent cell division defects. These results strongly suggest that *cdk-4* and *cyd-1* do not act exclusively upstream of *lin-35* Rb but most probably activate or inactivate additional targets.

***lin-35* Rb is not rate limiting for S-phase entry**

Because our results demonstrate that *lin-35* Rb acts as a negative regulator of cell division, we examined homozygous *lin-35* mutants in more detail for the presence of any defects in cell division. Using both Nomarski microscopy of live *lin-35* mutant larvae and fluorescence microscopy of fixed animals stained with propidium iodide (PI), we did not observe premature or additional cell divisions (data not shown). Alternatively, loss of *lin-35* might cause premature entry into S phase, which could be

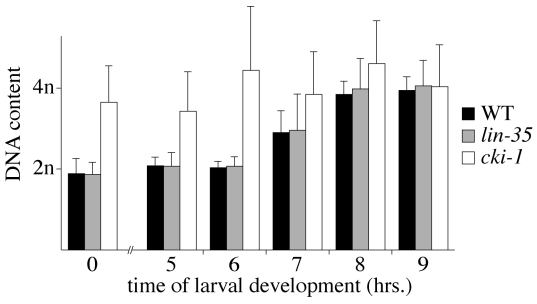

Fig. 5. *lin-35* is not rate limiting for S-phase entry. DNA content of intestinal cells of wild-type, *lin-35(n745)* and *cki-1(RNAi)* animals was determined at 1 hour intervals from the start of postembryonic development. Bars indicate mean of 10 intestinal nuclei±s.d.

compensated for by expanding later cell-cycle phases. We compared the timing of DNA replication in wild-type and *lin-35(n745)* mutant animals to examine this possibility. Using quantitative DNA measurements at different times of L1 development, we determined that the first round of DNA synthesis in the intestinal cells occurs between 6 and 8 hours of postembryonic development in wild-type animals. This timing was identical in *lin-35(n745)* mutants (Fig. 5). Thus, in contrast to mouse embryo fibroblasts that lack Rb family members, inactivation of *lin-35* Rb is not rate limiting in the normal regulation of S-phase initiation *in vivo*, and additional regulatory pathways probably control the timing of DNA replication in the absence of *lin-35*.

The Cip/Kip family members *cki-1* and *cki-2* cooperate with *lin-35* in G_1 regulation

Two observations suggest that *lin-35* Rb cooperates with other regulatory pathways in controlling progression through G_1 phase. First, loss of *lin-35* did not fully overcome the cell division defects of *cyd-1* and *cdk-4* mutants. Second, the timing of S-phase entry and cell division remained intact in mutants lacking *lin-35* function.

Several levels of control may converge at the level of Cyclin E/CDK2, as Cyclin E-CDK2 kinase activity is necessary and sufficient to induce S-phase in a variety of systems (Duronio and O'Farrell, 1995; Knoblich *et al.*, 1994; Ohtsubo *et al.*, 1995; Tsai *et al.*, 1993; van den Heuvel and Harlow, 1993). In addition to transcriptional suppression by pRb, Cyclin E/CDK2 kinases are also regulated by CDK inhibitors of the Cip/Kip family (Ekholm and Reed, 2000; Sherr and Roberts, 1999).The *C. elegans* genome contains two Cip/Kip family members, *cki-1* and *cki-2* (Feng *et al.*, 1999; Hong *et al.*, 1998). Several observations suggested that *cki-1* and possibly *cki-2* have conserved functions as CDK inhibitors. Ectopic expression of CKI-1 has been shown to arrest the cell cycle in G_1 phase (Hong *et al.*, 1998). Moreover, inactivation of *cki-1,* but not *cki-2,* by RNAi resulted in supernumerary divisions in various cell lineages (Feng *et al.*, 1999; Hong *et al.*, 1998). We confirmed the *cki-1* RNAi phenotype and observed low penetrant postembryonic cell divisions in *cyd-1* mutant animals after *cki-2* RNAi (see below and data not shown). Finally, CKI-1 was found to interact with CYD-1 and CYE-1 in two-hybrid assays (Materials and Methods). Together, these observations suggested that *cki-1* and *cki-2* (collectively referred to as *cki-1,2*) are *C. elegans* Cip/Kip family members with conserved functions in the regulation of G_1/S progression.

We examined if *cki-1,2* Cip/Kip activity is sufficient to control cell-cycle progression in the absence of *lin-35* Rb function. In contrast to *lin-35* inactivation, *cki-1* RNAi caused premature entry into S phase (Fig. 5). A significant number of intestinal nuclei obtained 4n DNA amounts even without stimulation of L1 development (Fig. 5, 0 hour). Interestingly, RNAi for *cki-1* alone or *cki-1* and *cki-2* together resulted in only a

single round of DNA replication in *cyd-1(he112)* mutant animals (Fig. 6A). Thus, inactivation of the *cki-1,2* inhibitors appears rate limiting for S-phase entry and allows one round of DNA duplication even in the absence of *cyd-1/cdk-4* function. However, subsequent rounds of DNA synthesis require the activity of *cyd-1* and *cdk-4* or inactivation of *lin-35* Rb. These results demonstrate that *cki-1,2* Cip/Kip and *lin-35* Rb contribute non-overlapping levels of control over the G_1/S transition.

Next we determined whether *lin-35* and *cki-1,2* cooperate in negatively regulating cell division. As shown above, inactivation of *lin-35* partly restored postembryonic cell division in *cyd-1* and *cdk-4* mutant animals (Figs 4C, 7B). Similarly, *cki-1,2* RNAi caused rescue of cell division in *cyd-1* mutants, resulting in an approximately normal number of P cell divisions and a limited number of intestinal divisions (Figs. 6B, 7C). Importantly, we observed dramatically increased numbers of cell divisions when inactivation of *lin-35* and *cki-1,2* were combined. Even in a *cyd-1* mutant background, this resulted in a total number of intestinal nuclei that far exceeded the number in wild-type animals (Figs. 6B, 7D). Double inactivation of *lin-35* Rb and *cki-1,2* Cip/Kip also caused a syner-

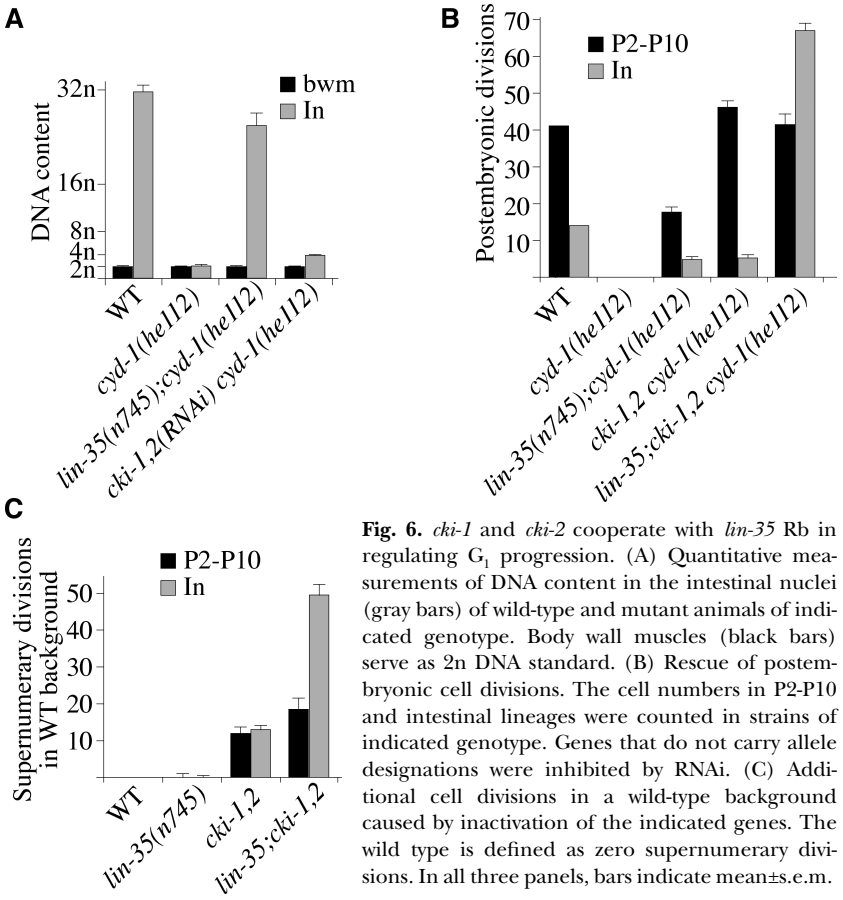

Fig. 6. *cki-1* and *cki-2* cooperate with *lin-35* Rb in regulating G_1 progression. (A) Quantitative measurements of DNA content in the intestinal nuclei (gray bars) of wild-type and mutant animals of indicated genotype. Body wall muscles (black bars) serve as 2n DNA standard. (B) Rescue of postembryonic cell divisions. The cell numbers in P2-P10 and intestinal lineages were counted in strains of indicated genotype. Genes that do not carry allele designations were inhibited by RNAi. (C) Additional cell divisions in a wild-type background caused by inactivation of the indicated genes. The wild type is defined as zero supernumerary divisions. In all three panels, bars indicate mean±s.e.m.

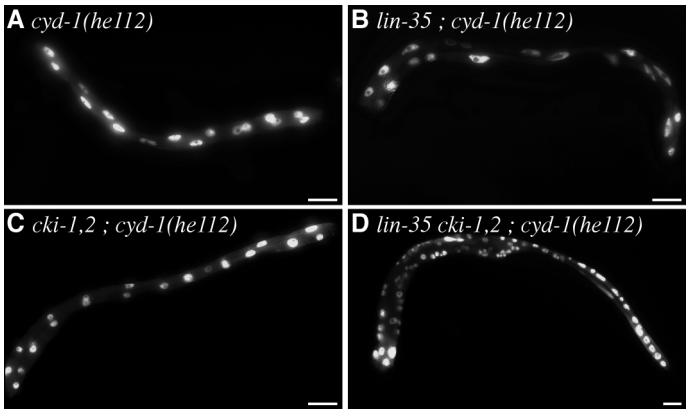

Fig. 7. Cip/Kip and Rb family members cooperate in regulating G_1 progression. Epifluorescent images demonstrating the number of intestinal divisions in (A) *cyd-1(he112); elt-2::GFP* mutant larvae and animals of the same genotype after (B) *lin-35* RNAi, (C) *cki-1,2* RNAi and (D) *lin-35; cki-1,2* double RNAi. Intestinal nuclei express the GFP under the control of the *elt-2* promoter region. Scale bars: 25 μm.

gistic increase in the number of supernumerary divisions in a wild-type background (Fig. 6C). Although the absolute effects vary between different cell lineages, *cki-1,2* Cip/Kip and *lin-35* Rb cooperate in regulating G_1/S phase progression.

DISCUSSION

Control of G_1 progression is crucial to the development of all eukaryotes. We followed a genetic approach in the nematode *C. elegans* to learn more about the pathways that regulate G_1 progression *in vivo*. A screen for positive regulators of G_1 progression identified a D-type cyclin and a CDK4/6 related kinase. Characterization of the mutant phenotypes confirmed the previous conclusion by Park and Krause (Park and Krause, 1999) that *cyd-1* and *cdk-4* are essential for entry into S-phase during postembryonic development. We used the mutant alleles of *cyd-1* and *cdk-4* to directly address whether Cyclin D and/or CDK4/6 act in a linear pathway with Rb family members *in vivo*. We found that although *lin-35* Rb is an important negative regulator of G_1 progression and probably acts downstream of *cyd-1* and *cdk-4*, *lin-35* does not provide the only level of regulation of S-phase entry. An additional level of control is contributed by the CDK inhibitors *cki-1* and *cki-2*, which were found to act in parallel to *lin-35*, and to cooperate with *lin-35* in controlling the G_1/S transition. Below we discuss our results in light of the existing knowledge about G_1 control in other animal systems.

Considering the nature of the screen, it is somewhat surprising that we identified only a D-type cyclin and CDK4/6 related kinase as essential positive regulators. Although few genes may be essential for both

rnr::GFP expression and cell division, there are several reasons why some regulators may have been missed. First, although multiple alleles of *cyd-1* and *cdk-4* were identified, it is unlikely that the screen was saturating. Moreover, mutation of some positive regulators may not have resulted in a prominent phenotype, owing to functional redundancy with other genes. In addition, a mutation may not result in a cell-cycle specific phenotype if the gene affected has additional essential functions. Finally, mutations will have been missed that cause a cell-cycle arrest before or after the L1 stage. In the presence of wild-type maternal product, the stage at which a mutant phenotype first becomes apparent is determined by the requirement for zygotic gene function. Although many *C. elegans* cell-cycle regulators display their mutant phenotype in the first larval stage (e.g. *ncc-1, lin-5, cul-1* Boxem *et al.*, 1999; Kipreos *et al.*, 1996; Lorson *et al.*, 2000), zygotic expression of other genes is required during embryonic development or after the first larval stage. For example, mutations in *cye-1*, the *C. elegans* Cyclin E homolog, result in late larval defects, although RNAi experiments revealed an essential function during embryogenesis (Fay and Han, 2000 M. B. and S. v.d.H., unpublished). We did not identify mutations in candidate positive regulators of the E2F/DP transcription factor families. Accordingly, the recent analysis of *efl-1* E2F and *dpl-1* DP mutations revealed that these genes are not generally needed for cell division in *C. elegans* (Ceol and Horvitz, 2001; Page *et al.*, 2001).

The Cyclin D kinase is essential for G_1 progression

A large number of observations have implicated Cyclin D-CDK4/6 kinases in G_1 control (Sherr, 1996; Sherr and Roberts, 1999). Most studies have documented the effects of 'gain of function' of kinase activity. For example, increased activity of D-type kinases is found in tumor cells, can shorten G_1 phase of cells in tissue culture and can overcome the G_1 arrest induced by ectopic pRb expression. Such observations do not establish whether or not these kinases are essential for cell cycle progression. Indirect evidence in support of an essential role has been provided by overexpressing the CDK4/6 inhibitor $p16^{INK4a}$ (Bruce *et al.*, 2000; Koh *et al.*, 1995; Lukas *et al.*, 1995; Medema *et al.*, 1995). This has been shown to arrest G_1 progression of pRb-positive cells, but not of cells lacking either pRb or p107 and p130. Mice nullizygous for Cyclin D1 or CDK4 develop to adults with growth defects (Fantl *et al.*, 1995; Rane *et al.*, 1999; Sicinski *et al.*, 1995; Tsutsui *et al.*, 1999). However, the effects have yet to be described of gene knockout of all three D-type cyclins, or of CDK4 and CDK6. The most complete inactivation of Cyclin D kinase activity may have been achieved in mice double null for p21 and p27 Cip/Kip, which act as assembly factors for CDK4/6-Cyclin D kinases (Cheng *et al.*, 1999; LaBaer *et al.*, 1997). Double inactivation of p21 and p27 has been found to reduce CDK4/6 kinase activity below the level of detection, yet these double mutant animals do not show

cell-division defects (Cheng *et al.*, 1999). Such results have challenged the prevalent view about the requirement for Cyclin D kinase activity.

C. elegans and *Drosophila* are thus far the only organisms in which the effects of complete loss of Cyclin D-dependent kinase activity have been studied. Inactivation of the sole CDK4/6-related kinase in *Drosophila* did not affect cell division in a general way (Datar *et al.*, 2000; Meyer *et al.*, 2000). Homozygous *Cdk4* mutant flies develop into small adults with reduced fertility (Meyer *et al.*, 2000). The small size did not appear to be caused by a decrease in cell numbers. Rather, based upon the analysis of *Cdk4* mutants and ectopic expression of CycD-Cdk4, the primary role of CycD-Cdk4 appears to be stimulation of cell growth in *Drosophila* (Datar *et al.*, 2000; Meyer *et al.*, 2000). By contrast, the cell-division defects in the *C. elegans cyd-1* and *cdk-4* mutants precede a detectable growth defect and first appear in late embryogenesis before growth takes place. Thus, the rate limiting function of Cyclin D-CDK4/6 kinases may vary between species, demonstrating the value of using multiple model organisms in studying gene function.

***lin-35* Rb probably acts downstream of *cdk-4/cyd-1* in cell-cycle control**

Upon inactivation of the retinoblastoma family member *lin-35* Rb, cells were able to complete multiple rounds of S phase in the apparent absence of CYD-1/CDK-4 activity. This clearly establishes *lin-35* Rb as a negative regulator of S phase which acts downstream of or in parallel to *cyd-1/cdk-4*. Previously, *lin-35* was identified as a member of a set of genes that negatively regulate vulval cell fate (Ferguson and Horvitz, 1989; Lu and Horvitz, 1998). These genes are known as the 'synthetic multivulva' (synMuv) genes that form two functionally redundant classes. Inactivation of both a class A and a class B synMuv gene causes inappropriate induction of vulval fates, resulting in a Multivulva phenotype (Ferguson and Horvitz, 1985; Ferguson and Horvitz, 1989). Genetic epistasis experiments have shown that the synMuv genes act to antagonize a receptor tyrosine kinase/Ras-mediated signaling pathway (Lu and Horvitz, 1998). A role in cell-division has not been described previously for the class B synMuv gene *lin-35* Rb. *lin-35* null mutants are viable and appear to develop normally (Lu and Horvitz, 1998). In addition, the absence of *lin-35* function did not affect the timing of S-phase entry. These findings are surprising as *lin-35* is the only member of the Rb family in *C. elegans*, and loss of Rb is lethal in mice (Jacks *et al.*, 1992) as well as in *Drosophila* (Du and Dyson, 1999). Moreover, mouse embryonic fibroblasts that lack all three Rb family members show severe cell-cycle defects in tissue culture (Dannenberg *et al.*, 2000; Sage *et al.*, 2000). Interestingly, in chimeric mouse experiments, adult mice with largely normal tissues were found to contain high percentages of $Rb^{-/-}$ cells (Robanus Maandag *et al.*, 1994; Williams *et al.*, 1994). Thus, the developmental requirement for a functional *Rb* gene may be limited to specific tissues or cell types.

***lin-35* Rb and *cki-1* Cip/Kip cooperate in G_1/S control**

Our results demonstrate that in the absence of *lin-35* Rb function additional levels of control are sufficient to maintain the correct timing of S phase in *C. elegans*. Based on results in other systems, we reasoned that such controls are likely to involve inhibitors of the Cip/Kip family. Indeed, several results indicate that *cki-1,2* Cip/Kip and *lin-35* Rb cooperate in controlling the G_1/S transition in *C. elegans*. The strongest argument for cooperation between *cki-1,2* and *lin-35* is the observed synergistic effect of double inactivation on supernumerary cell divisions. Most strikingly, animals contained on average 84 intestinal nuclei following inactivation of *cki-1,2* as well as *lin-35*, while adult wild-type animals, *lin-35* mutants, and *cki-1,2(RNAi)* animals averaged 34, 34 and 47 nuclei, respectively. Such increased numbers of intestinal divisions have not been reported previously in *C. elegans*. That *cki-1* and *lin-35* act at least in part in parallel pathways is further indicated by their distinct loss-of-function phenotypes. Loss of *lin-35* does not lead to precocious S phase but does allow multiple rounds of DNA replication in *cyd-1* or *cdk-4* mutant animals. By contrast, inactivation of *cki-1* by RNAi results in premature S phase, yet permits only a single round of DNA synthesis in *cyd-1* and *cdk-4* mutants. These results agree well with those obtained for *dacapo,* a Cip/Kip inhibitor in *Drosophila* (de Nooij *et al.*, 1996; Lane *et al.*, 1996). The *dacapo* mutant phenotype has been proposed to result from failure to inactivate residual Cyclin E kinase (de Nooij *et al.*, 1996). For further rounds of cell division, Cyclin E needs to be transcribed, which requires inactivation of RBF/E2F-mediated transcriptional repression. We expect that similar mechanisms explain the cooperative effects of *cki-1* and *lin-35* Rb in *C. elegans*.

Several observations in mammalian systems also suggest cooperation. In tissue culture, $p21^{-/-}$;$pRb^{-/-}$ mouse cells are more defective in G_1 control than cells lacking either single gene (Brugarolas *et al.*, 1998). Moreover, double inactivation of genes in both pathways increases tumor formation in mouse models (Brugarolas *et al.*, 1998; Franklin *et al.*, 1998; Park *et al.*, 1999). As p21 is an important downstream target of the p53 tumor suppressor, cooperation between p21 and pRb in cell cycle control may contribute to the strong selective pressure for dual inactivation of p53 and pRb in human cancer. Thus, although control by Cip/Kip inhibitors may be more rate limiting in *C. elegans*, cooperation between Cip/Kip and pRb family members in G_1/S control is probably shared among metazoans.

Additional functions of Cyclin D-CDK4/6 kinases

The absence of pRb family proteins in *lin-35* putative null mutants was not sufficient to fully overcome the requirement for a Cyclin D-dependent kinase in *C. elegans*. This indicates that *cyd-1* and *cdk-4* do not act solely to inactivate *lin-35*. A second activity described for Cyclin D-CDK4/6 complexes is sequestration of Cip/Kip inhibitors, which

allows activation of Cyclin E/CDK2 complexes (Sherr and Roberts, 1999). It is possible that sequestering CKI-1 and CKI-2 is the detected additional function of the CYD-1/CDK-4 kinase. Consistent with this idea, we found that CYD-1, as well as CYE-1, interacts with CKI-1 in the two hybrid system. Interestingly, the single *Drosophila* Cip/Kip family member Dacapo does not bind to CycD-Cdk4 (Meyer *et al.*, 2000). Possibly, alternative ways to control Cip/Kip activity have been developed in *Drosophila*, which may explain the reduced role of the Cyclin D-CDK4/6 kinase in cell-cycle progression.

MATERIALS AND METHODS

Culture conditions and strains

We used the wild-type strains N2 and RW7000 and the following mutations, descriptions of which can be found elsewhere (Riddle *et al.*, 1997):
LGI: *dpy-5(e61)*, *lin-35(n745* and *n2239)* (Lu and Horvitz, 1998), *unc-29(e403)*.
LGII: *dpy-10(e128)*, *rol-1(e91)*, *cyd-1(he112* and *he116)* (this study).
LGX: *lon-2(e678)*, *unc-9(e101)*, *cdk-4(he109*, *he110*, *he111* and *gv3)* (this study) (Park and Krause, 1999).
Deficiency: *heDf1*.
Rearrangements: *mnC1(II)* (Wood and the community of *C. elegans* researchers, 1988).
Integrated arrays: *maIs103[rnr::GFP unc-36(+)]X* (Hong *et al.*, 1998), *rtIs14[elt-2::GFP; osm-10::HT150QJIV* (a gift from P. W. Faber and A. Hart).

Screen for positive regulators of G_1/S progression

Animals of genotype *unc-36(e251);maIs103[rnr::GFP unc-36(+)]*(Hong *et al.*, 1998), expressing GFP controlled by *ribonucleotide reductase* (*rnr*) promoter sequences, were mutagenized with 25 mM ethylmethanesulfonate as described (Brenner, 1974). Individual mutagenized F1 animals were picked to plates and their progeny examined for the presence of 1/4 sterile uncoordinated animals. Such mutants were further examined for absence of postembryonic cell divisions and GFP expression. Candidate mutations identified from ~10,000 haploid genomes were recovered from heterozygous siblings and mapped to chromosomes by PCR, making use of primers based on polymorphic Sequence-Tagged Sites in the RW7000 Bergerac strain (Williams *et al.*, 1992). Two mutations (*he112* and *he116*) were placed on chromosome II and three mutations (*he109*, *he110* and *he111*), as well as the deletion *heDf1*, were placed on the X chromosome. Standard two- and three-factor mapping with *dpy-10(e128)* and *rol-1(e91)* or *lon-2(e678)* and *unc-9(e101)* was performed for further mapping. DNA sequence analysis revealed molecular lesions in *cyd-1* (*he112*, Q292->stop) and *cdk-4* (*he110*, E85->K; *he111*: W208-> STOP; *he109*: Q292-> STOP). PCR and Southern blotting experiments revealed that *he116* and *heDf1* are deletions.

Quantitation of DNA and cell division

Quantitative determination of DNA content was performed essentially as described before (Boxem *et al.*, 1999), by measuring the pixel intensity of serial *z*-sections taken on a confocal microscope (Zeiss) of animals stained with propidium iodide. The number of cell divisions was determined by counting nuclei in fixed specimens. The daughter cells of P2 to P10 as well as the intestinal nuclei could be recognized unambiguously and are therefore used in the figures. Owing to partial rescue of cell division, *cyd-1* and *cdk-4* mutants were recognizable and formed 1/4 of the total offspring. Strains carrying the *elt-2::GFP* reporter were used to recognize intestinal nuclei in the experiments shown in Figs 6 and 7.

Two-hybrid assays
A full-length *cki-1* cDNA was obtained from *C. elegans* cDNA by PCR using primers 5'-gggg-gaccactttgtacaagaaagctgggtgtatggagagcatgaagatcg and 5'-ggggacaagtttgtacaaaaaagcaggct-tgtcttctgctcgtcgttgc. Sequence analysis confirmed the obtained PCR product corresponded to the wild-type *cki-1* sequence. The *cki-1* cDNA was cloned into pPC97 and used as bait in a two-hybrid experiment as described (Vidal, 1997). CYE-1 and CYD-1 were each isolated independently five times as a CKI-1 interacting protein, out of a total of 2×10^6 yeast colonies transformed with the *cki-1* bait construct.

Timing of DNA replication
N2 animals were injected with *lin-35* dsRNA or *cki-1* dsRNA, and allowed to produce progeny for 24 hours. Eggs produced in the next 24 hours at 15°C were hatched in the absence of food, which results in a developmental arrest immediately after hatching. Subsequently, synchronous development was induced by transferring the arrested larvae to fresh agar plates containing *E. coli* bacteria. Developing larvae were fixed in Carnoys fixative at 1 hour intervals and stained with the DNA stain propidium iodide. DNA contents of 10 In cells were determined as described above.

Growth rate of *cyd-1* mutants
Embryos were collected by hypochlorite treatment of gravid *cyd-1(he112)/mnC1* adults and allowed to hatch in S-medium without food (Wood and the community of *C. elegans* researchers, 1988). Synchronous L1 development was initiated 24 hours later by transferring the starved L1 animals to agar plates containing *E. coli* bacteria. We determined the total body size of five *cyd-1* mutants and five heterozygous siblings at various times, using the size measurement function of the Openlab software package. The software was calibrated using a size standard slide.

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Chapter 4

C. elegans class B synthetic Multivulva genes act in G_1 regulation

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ABSTRACT

The single Rb family member in *C. elegans*, *lin-35*, was originally identified as a member of the synthetic Multivulva (synMuv) genes (Lu and Horvitz, 1998). These genes form two redundant classes, A and B, that repress ectopic vulval cell fate induction (Ferguson and Horvitz, 1985; Ferguson et al., 1987). Recently, we demonstrated that *lin-35* Rb also acts as a negative regulator of G_1 progression, and likely is the major target of *cyd-1* Cyclin D and *cdk-4* CDK4/6 (Boxem and van den Heuvel, 2001). To identify additional G_1 control genes, we examined other synMuv genes and observed cell-cycle functions for several class B genes. We found that *efl-1* E2F negatively regulates cell-cycle entry, while *dpl-1* DP appears to act both as a positive and negative regulator. In addition, we identified a negative G_1 regulatory function for *lin-9* ALY, as well as *lin-15*B and *lin-36*, which encode novel proteins. Inactivation of *lin-35* Rb, *efl-1* or *lin-36* allowed S phase entry in the absence of *cyd-1/cdk-4* and increased ectopic cell division when combined with *cki-1* Cip/Kip RNAi. These data are consistent with *lin-35* Rb, *efl-1* and *lin-36* acting in a common pathway or complex that negatively regulates G_1 progression. In contrast, *lin-15*B appeared to act in parallel to *lin-35*. Our results demonstrate the potential for genetic identification of novel G_1 regulators in *C. elegans*.

RESULTS AND DISCUSSION

The tumor suppressor pRb acts as a negative regulator of cell-cycle entry. This function is thought to require interaction with sequence-specific transcription factors, such as E2F/DP heterodimers, that attract pRb to the promoter regions of genes required for S phase. In turn, pRb likely recruits chromatin remodeling complexes, such as histone deacetylases (HDACs), that promote transcriptional repression. As many protein associations have been described for pRb, it remains unclear which interactions are of critical importance for pRb-mediated G_1 regulation *in vivo* (Morris and Dyson, 2001). Demonstration of such *in vivo* functions requires genetic studies in model organisms.

The nematode *Caenorhabditis elegans* contains a single Rb-related gene, *lin-35* (Lu and Horvitz, 1998). This gene was identified originally as a synthetic Multivulva (synMuv) gene that prevents ectopic vulval cell fate specification (Ferguson and Horvitz, 1985; Ferguson *et al.*, 1987). Inactivation of *lin-35* Rb does not cause obvious cell-cycle defects, since cyclin-dependent kinase (CDK) inhibitor(s) of the Cip/Kip family are rate limiting for G_1/S progression (Boxem and van den Heuvel, 2001). However, a cell-cycle role for *lin-35* Rb became evident when examined in combination with inactivation of other cell-cycle regulators (Boxem

*	*lin-8, lin-15*A, *lin-38, egr-1* and *egl-27* have been classified as class A synMuv genes, while *lin-9, lin-15*B, *lin-35, lin-36, lin-37, lin-53, dpl-1, efl-1, tam-1, hda-1, let-418* and possibly *chd-3* act as class B genes (however, see Solari and Ahringer, 2000). Although *efl-2* does not act as a synMuv gene it was included because of its homology to E2F.
†	To accomplish loss of function, we made use of mutant alleles, RNAi and TSA (Trichostatin A, histone deacetylase inhibitor) as indicated. The following mutant alleles were used, descriptions of which can be found in (Riddle *et al.*, 1997), or cited references: *lin-35(n745, n2239)* I (Lu and Horvitz, 1998), *lin-53(n833)* I (Lu and Horvitz, 1998), *lin-8(n111)* II, *lin-38(n751)* II, *cyd-1(he112)* II (Boxem and van den Heuvel, 2001), *lin-36(n766)* III, *lin-37(n758)* III, *cdk-4(gv3)* X (Park and Krause, 1999), *lin-15*A*(n767)* X, *lin-15*B*(n374)* X (Ferguson and Horvitz, 1989).
‡	(+): see text and figures for quantitative results, (-): cell division, endoreduplication or *rnr::GFP* expression were identical to *cyd-1(he112).*
**	Rescue of *rnr::GFP* was performed by RNAi. Only genes that have been cloned were tested.
††	Double RNAi for *cki-1* and *cki-2* was performed, although the effects of *cki-2(RNAi)* were minimal (Boxem and van den Heuvel, 2001).
1	*let-418* has also been named *chd-4* and *evl-11,* see references in (von Zelewsky *et al.*, 2000). RNAi causes a developmental arrest in the L1 stage.
2	As injection of dsRNA into adults resulted in embryonic lethal progeny, two additional methods were applied to obtain larval phenotypes. Synchronized L1 larvae were soaked in dsRNA for 24 hours, prior to stimulation of larval development by feeding. In addition, dsRNA was injected into *rde-1(ne219)* RNAi resistant animals (Tabara *et al.*, 1999), followed by mating with *cyd-1*/+ males. The males provide a wild type copy of the *rde-1* gene, which upon zygotic expression allows for a late embryonic or larval RNAi effect.
3	*egl-27* RNAi causes embryonic lethality and early L1 arrest, and was not pursued further.
4	See supplementary material.
5	*dpl-1* RNAi specifically inhibits *rnr::GFP* expression.

Table 1. Cell cycle regulatory function of *C. elegans* synMuv genes

Gene*	Homolog	Methods of inactivation†	Suppression of *cyd-1* phenotype‡			Cooperation with *cki-1,2*††
			cell division	DNA synthesis	*rnr::GFP* expression**	
lin-8	not cloned	mutant	-	-	ND	ND
lin-9	*Dm* aly	mutant/RNAi	+	+	ND[4]	-
*lin-15*A	novel	mutant/RNAi	-	-	-	ND
*lin-15*B	novel	mutant/RNAi	+	-	-	-
lin-35	Rb	mutant/RNAi	+	+	+	+
lin-36	novel	mutant/RNAi	+	+	-[4]	+
lin-37	novel	mutant	-	-	ND	ND
lin-38	not cloned	mutant	-	-	ND	ND
lin-53	RbAP46/48	mutant/RNAi[2]	-	-	ND	ND
dpl-1	DP	RNAi	±	+	-[5]	+
efl-1	E2F	RNAi	+	+	+	+
efl-2	E2F	RNAi	-	-	-	ND
tam-1	RING finger/B box	RNAi	-	-	-	ND
hda-1	HDAC	RNAi[2]/TSA	-	-	ND	ND
chd-3	Mi-2	RNAi	-	-	-	ND
let-418[l]	Mi-2	RNAi	-[1]	ND[1]	+	ND
egr-1	MTA	RNAi	-	-	-	ND
egl-27	MTA	RNAi	ND[3]	ND[3]	ND[3]	ND

and van den Heuvel, 2001). Loss of function of *cyd-1*, the only D-type cyclin gene in *C. elegans*, or of *cdk-4*, the single CDK4/6 kinase gene, causes arrest of postembryonic cell divisions in G_1 phase (Boxem and van den Heuvel, 2001; Park and Krause, 1999). Inactivation of *lin-35* Rb in *cyd-1* or *cdk-4* mutants overcomes this cell-cycle arrest (Boxem and van den Heuvel, 2001). Moreover, inactivation of *lin-35* Rb together with *cki-1,2* Cip/Kip causes a synergistic increase in the number of ectopic cell divisions (Boxem and van den Heuvel, 2001) (see *cki-1,2* footnote Table 1). These results established that *lin-35* Rb acts as a negative regulator of G_1 progression, most likely downstream of *cyd-1/cdk-4* (Boxem and van den Heuvel, 2001).

To identify additional G_1 regulators, we examined whether synMuv genes other than *lin-35* Rb function in cell-cycle regulation. We monitored whether inactivation of synMuv genes in a *cyd-1* mutant background restored expression of an S-phase reporter (*rnr::GFP*, Hong *et al.*, 1998), whether it allowed DNA synthesis, and whether it rescued the cell division arrest (see Supplementary Experimental Procedures). In addition, we examined the effects of double inactivation of synMuv genes and *lin-35* or *cki-1,2* to further examine functional similarity with *lin-35* Rb. Mutations in *lin-35* Rb pathway genes that act as G_1/S inhibitors are expected to increase the number of ectopic cell divisions caused by *cki-1,2* inactivation, but should not affect the *lin-35* mutant phenotype. The results of these assays are summarized in Table 1 and discussed in detail below.

No apparent G_1 function for synMuv class A genes

lin-35 Rb is a class B synMuv gene which acts redundantly with class A synMuv genes in vulval fate determination. Interestingly, we did not observe a role for class A genes in cell-cycle control (Table 1). Mutations in the class A genes *lin-8*, *lin-15*A and *lin-38* did not restore cell division or S phase entry in *cyd-1* mutant animals. Moreover, the partial rescue of postembryonic cell divisions in *lin-35(n745);cyd-1(he112)* double mutant animals was not affected by further introduction of the *lin-8(n111)* or *lin-15*A*(n765)* mutations or by *lin-15*A RNAi (not shown). Therefore, the observed functional redundancy between class A and class B genes in vulval fate determination does not appear to apply to G_1 regulation.

Regulation of G_1 progression by *efl-1* E2F and *dpl-1* DP

Two of the class B synMuv genes are *C. elegans* homologs of E2F and DP (Ceol and Horvitz, 2001). RNAi of *efl-1* E2F caused a cell-cycle phenotype that was strikingly similar to the *lin-35* Rb phenotype (Table 1, Fig. 1). Inactivation of *efl-1* resulted in multiple rounds of DNA synthesis in the intestinal nuclei (Int) of *cyd-1(he112)* mutant larvae (average 20.5 n±2.1 s.e.m, Fig. 1A). RNAi of *efl-1* also allowed expression of the *rnr::GFP* S-phase marker in the postembryonic blast cells of *cyd-1* mutants (Fig. 1B). In addition, *efl-1* RNAi partially restored postembry-

onic cell divisions (Fig. 1C). Finally, RNAi for *efl-1* and *cki-1,2* simultaneously resulted in a far greater number of intestinal nuclei than inactivation of either *efl-1* or *cki-1,2* alone (up to 70 ectopic divisions, compared to at most 3 and 26, respectively) (Fig. 1D). This effect was observed both in a wild-type and a *cyd-1* mutant background, and closely resembled the effect of simultaneous inactivation of *lin-35* and *cki-1,2*. As expected, *efl-1* RNAi in a *lin-35(n745)* mutant background did not

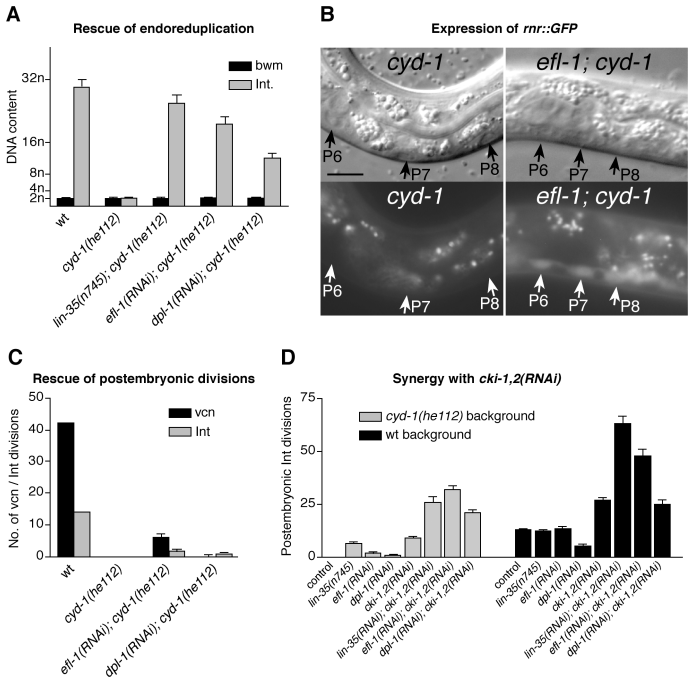

Fig. 1. *dpl-1* DP and *efl-1* E2F regulate G_1 progression. (A) Loss of *efl-1* or *dpl-1* results in multiple rounds of DNA synthesis in *cyd-1* mutant larvae, as demonstrated by quantitative measurements of DNA content. The total DNA content of 10 intestinal cells (Int) was determined for animals of indicated genotypes as described previously (Boxem and van den Heuvel, 2001). Int cells in wild-type animals undergo endoreduplication and accumulate a 32n DNA content. Body wall muscle cells (bwm) are used as a 2n standard. (B) Loss of *efl-1* restores *rnr::GFP* expression in *cyd-1* mutants. Nomarski (top) and epifluorescent (bottom) images show several "P" cells of the ventral cord precursor lineage expressing *rnr::GFP* in an *efl-1(RNAi); cyd-1(he112)* mutant animal (right), but not in a *cyd-1* mutant animal (left). Scale bar corresponds to 10 μm. (C) Loss of *efl-1* and *dpl-1* partially restores cell division in *cyd-1* mutant larvae. The number of ventral cord neurons (vcn) formed during larval development was counted between the retrovesicular ganglion and P10.p, as well as the number of intestinal nuclear divisions (Int) for 10 animals of each indicated genotype. Wild-type numbers correspond to those reported previously (Sulston and Horvitz, 1977) (D) *efl-1* and *dpl-1* cooperate with *cki-1* and *cki-2* in negatively regulating G_1 progression. Postembryonic intestinal divisions were determined for 10-20 animals of indicated genotypes. An *elt-2::GFP* reporter was used to mark intestinal nuclei. To distinguish *cyd-1* mutants from siblings, *cyd-1* was marked *in trans* with *mIn1* which contains an integrated array expressing GFP in the pharynx, controlled by the *myo-2* promoter (Edgley and Riddle, 2001). In all panels, bars indicate mean± s.e.m.

cause any increase in postembryonic cell divisions (not shown). Together, these data indicate that *efl-1* acts as a negative regulator of cell-cycle entry, either downstream of or in parallel to *cyd-1*. Based on the similarity in phenotype, most of the G_1 control function of *lin-35* Rb appears to be mediated in concert with *efl-1* E2F.

The presumed protein partner of EFL-1 E2F is encoded by the synMuv B gene *dpl-1* DP (Ceol and Horvitz, 2001). Interestingly, *dpl-1* showed aspects of a positive regulator as well as a negative regulator of S phase entry. In *cyd-1(he112)* mutant larvae, *dpl-1* RNAi resulted in several rounds of DNA replication (Fig. 1A), and restored cell division at a level that was limited (Fig. 1C) but synergistically enhanced by simultaneous RNAi of *cki-1,2* (Fig. 1D, left). On the other hand, when examined in a wild-type background, *dpl-1* inactivation resulted in a decreased number of ventral cord and intestinal divisions (Fig. 1D, right, Ceol and Horvitz, 2001). Moreover, *dpl-1* RNAi caused repression of *rnr::GFP* expression in wild-type animals (see Supplementary Material). Together, these results indicate that *dpl-1* acts both as a negative and a positive regulator of G_1 progression.

Studies in other systems demonstrated that some E2F/DP heterodimeric transcription factors repress E2F responsive genes in cooperation with Rb, while others are predominantly transcriptional activators (Frolov *et al.*, 2001; Trimarchi and Lees, 2002). Our results indicate that the same model applies to developmental cell-cycle control in *C. elegans*. We wished to examine whether the only other *C. elegans* E2F-like gene, *efl-2* (Ceol and Horvitz, 2001), acts as a transcriptional activator. However, we did not observe suppression of *rnr::GFP* expression following *efl-2* RNAi, and double RNAi for *efl-1* and *efl-2* was found to resemble *efl-1* RNAi rather than *dpl-1* RNAi. It remains unclear whether the lack of phenotype was caused by ineffective RNAi or lack of *efl-2* requirement. Thus, although the partly opposite effects of *efl-1* and *dpl-1* RNAi suggest the presence of an activating E2F in *C. elegans*, no such E2F has yet been identified.

Our results are the first demonstration of a general cell-cycle function of *C. elegans efl-1* E2F. In combination with the observed formation of a trimeric complex of EFL-1, DPL-1 and LIN-35 *in vitro* (Ceol and Horvitz, 2001), our results support the model that EFL-1 and DPL-1 heterodimers recruit LIN-35 Rb for transcriptional repression of S phase genes (Fig. 3). These observations stress the importance of specific E2Fs as inhibitors of G_1 progression.

NuRD Components and G_1 regulation

Several synMuv gene products are structurally similar to components of the nucleosome remodeling and histone deacetylase (NuRD) complex (Ahringer, 2000; Lu and Horvitz, 1998; Solari and Ahringer, 2000; von Zelewsky *et al.*, 2000). LIN-35 can interact with the NuRD components

HDA-1 and LIN-53 *in vitro* (Lu and Horvitz, 1998). These results indicated that recruitment of the NuRD complex is critical to the role of *lin-35* Rb in vulval development. To address if NuRD recruitment is also critical in G_1 regulation, we examined homologs of the histone deacetylase, RbAp46/48 (Rb-associated protein), MTA1 and Mi-2 subunits (Table 1). In general, the cell-cycle effects of *lin-35* Rb inactivation were not reproduced by loss of function of NuRD genes (see Supplementary Material). The interpretation of these results is complicated by redundancies and pleotropic functions of NuRD genes, and the possibility of incomplete loss of function in our experiments. We consider two possible explanations for the lack of cell-cycle phenotype: NuRD components may not be generally required for cell-cycle inhibition, or a lower level of NuRD function may be needed for cell-cycle inhibition than for vulval development.

The synMuv class B genes *lin-9*, *lin-15*B and *lin-36* encode novel negative G_1 regulators

In addition to cognate components of the mammalian Rb regulation pathway, we tested five synMuv class B genes with unknown molecular functions: *lin-9, lin-15*B, *lin-36, lin-37,* and *tam-1.* Examination of *lin-37(n758); cyd-1(he112)* and *cyd-1(he112); tam-1(RNAi)* double mutant larvae did not reveal a G_1/S control function for the *lin-37* and *tam-1* genes (Table 1). However, three other synMuv B genes, *lin-9, lin-15*B and *lin-36,* displayed a cell-cycle phenotype.

A surprising result was obtained for *lin-15*B: although *lin-15*B and *lin-35* Rb act in a common synMuv B pathway, these genes do not appear to act in the same G_1 control pathway. *lin-15*B RNAi weakly rescued the postembryonic cell divisions in *cyd-1(he112)* mutant larvae (Fig. 2A; 4.7 P cell divisions, no Int divisions, n = 10). However, in double inactivation experiments, cooperation was observed between *lin-15*B and *lin-35* Rb, rather than *lin-15*B and *cki-1,2* Cip/Kip (Fig. 2D, not shown). Loss of either *lin-15*B or *lin-35* alone did not cause additional cell division, while *lin-35; lin-15*B double RNAi resulted in supernumerary cell divisions (Fig. 2D, on average 9.5 extra Int divisions, n = 10 animals). This synergistic effect could result from inactivation of two redundant pathways, or from combining partial loss of function of two genes within the same pathway. To distinguish between these possibilities, we used the strong or complete loss-of-function alleles *lin-35(n745)* and *lin-15*B*(n374),* and obtained similar results (Fig. 2D). Therefore, we conclude that *lin-35* and *lin-15*B act in at least partially non-overlapping pathways for G_1 control.

Inactivation of *lin-9* or *lin-36* restored several rounds of endoreduplication in *cyd-1(he112)* mutant larvae (Fig. 2A). In addition, *lin-36* loss-of-function caused significant rescue of postembryonic cell division in *cyd-1* mutants, while *lin-9* loss-of-function allowed an occasional division (Fig. 2B). Introduction of the strong loss-of-function alleles

lin-36(n766) and *lin-9(n942)* caused similar results as RNAi, but rescue was not obtained with the hypomorphic allele *lin-9(n112)* (Fig. 2A,B and data not shown). Inactivation of *lin-9* or *lin-36* restored cell division and endoreduplication in *cdk-4(gv3)* and *cyd-1(he112)* mutant larvae to the same extent (Fig. 2A,B). These results indicate that *lin-9* and *lin-36* act as negative regulators of G_1 progression downstream of or in parallel to *cyd-1* Cyclin D/*cdk-4* CDK4/6.

To examine whether *lin-9* and *lin-36* may act in the *lin-35* Rb G_1 control pathway, we created double loss-of-function combinations with

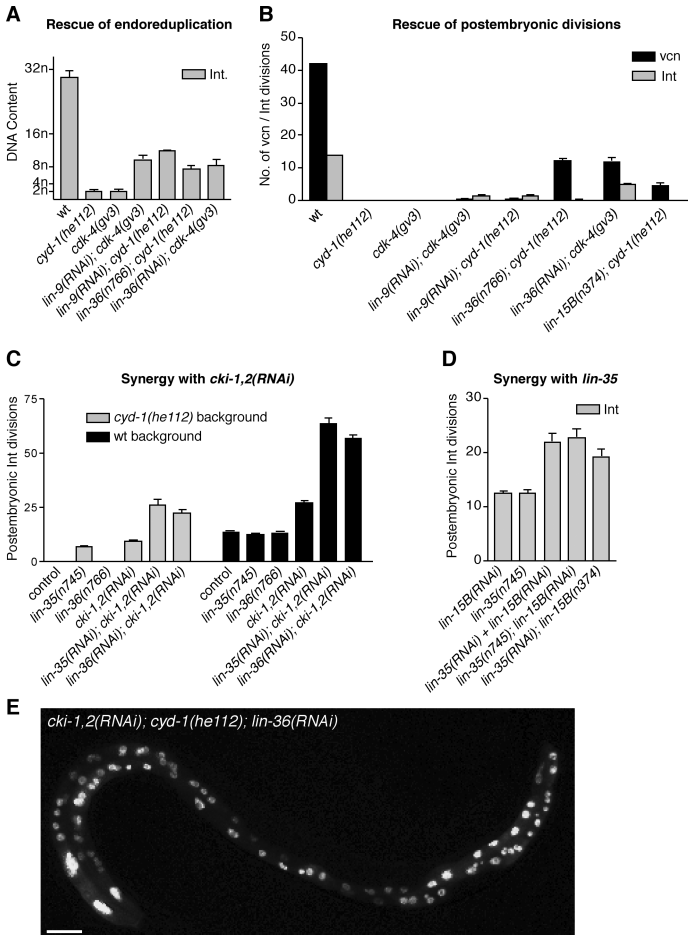

Fig. 2. Cell-cycle roles for *lin-9*, *lin-15*B and *lin-36*. (A) Loss of *lin-9* or *lin-36* results in multiple rounds of endoreduplication in *cyd-1* or *cdk-4* mutant larvae. (B) Loss of *lin-9*, *lin-15*B or *lin-36* partly rescues *cyd-1* cell division defects. (C) *lin-36* cooperates with *cki-1,2* in negatively regulating G_1 progression. Postembryonic intestinal divisions were determined for 10-20 animals of indicated genotypes. (D) *lin-15*B cooperates with *lin-35* Rb. Postembryonic intestinal divisions were determined for 10-20 animals of indicated genotypes. (E) Example of the dramatically increased intestinal divisions in a *cki-1,2(RNAi); cyd-1(he112); lin-36(RNAi)* animal. See the legend of Fig. 1 for methods. In panels C, D and E, an *elt-2::GFP* reporter was used to mark intestinal nuclei. Error bars indicate mean± s.e.m.

lin-35(n745) and *cki-1,2(RNAi)*. Inactivation of *lin-9* did not increase the number of intestinal divisions when combined with inactivation of either *lin-35* or *cki-1,2* (data not shown). However, RNAi for *lin-36* and *cki-1,2* together resulted in a far greater number of postembryonic cell divisions than inactivation of *cki-1,2* alone, both in a *cyd-1(he112)* mutant (Fig. 2C, left) and in a wild-type background (Fig. 2C, right). This effect closely resembled *lin-35*; *cki-1,2* double inactivation. Single RNAi of *lin-35* or *lin-36* did not cause supernumerary cell divisions, while *cki-1,2* RNAi resulted in on average 13 extra divisions in the intestinal lineage. Simultaneous inactivation of *cki-1,2* with either *lin-36* or *lin-35* resulted in on average 59 and 52 extra postembryonic intestinal divisions, respectively (Fig. 2C,E). In contrast, *lin-35; lin-36* double mutants behaved similar to *lin-35* single mutants in all assays (not shown). Thus, *lin-35* Rb and *lin-36* are both required for normal vulval fate determination as well as cell-cycle regulation, and both genes likely act downstream of *cyd-1/cdk-4* and parallel to *cki-1,2* Cip/Kip. These results are all consistent with *lin-36* acting in the *lin-35* Rb pathway as an inhibitor of G_1 progression (Fig. 3).

The sensitized genetic backgrounds that previously allowed us to detect the G_1 control function of *lin-35* Rb (Boxem and van den Heuvel, 2001) were used here to identify cell-cycle regulatory functions of other synMuv genes. Our results demonstrate several important aspects of G_1 regulation in *C. elegans*. We identified for the first time a role for *efl-1* E2F and *dpl-1* DP in regulating G_1 progression. This validates our assays and emphasizes the conservation of the Rb pathway for G_1 control in *C. elegans*. In addition, we detected novel roles as cell-cycle regulators for

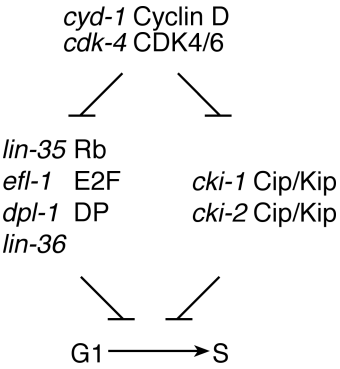

Fig. 3. Model for the regulation of S phase entry in *C. elegans*. Activity of a kinase complex consisting of CYD-1 Cyclin D and CDK-4 CDK4/6 is essential for entry into S phase (Boxem and van den Heuvel, 2001). This kinase appears to exert at least two functions in cell-cycle control: it antagonizes LIN-35 Rb, EFL-1, DPL-1 and LIN-36, possibly by phosphorylation of one or more of these components. In addition, it inhibits CKI-1 (and CKI-2) Cip/Kip family members, possibly by sequestration. In analogy with other systems, the *lin-35* Rb branch of the pathway likely inhibits transcription of genes required for the G_1/S transition. CKI-1,2 Cip/Kip likely inhibits a Cyclin E/CDK complex through direct protein interaction. Cell-cycle roles were also observed for *lin-9* and *lin-15*B. *lin-9* did not act synergystically with *lin-35* Rb or *cki-1,2* Cip/Kip, and was therefore not placed in a pathway. *lin-15*B acts in parallel to *lin-35* Rb, and could therefore potentially act in the *cki-1,2* Cip/Kip pathway. However, *lin-15*B mutants do not display the additional cell divisions found in *cki-1,2(RNAi)* animals, and *cki-1,2(RNAi)* was not observed to cause a synMuv phenotype. It is therefore also possible that *lin-15*B acts in a third pathway. DPL-1 also acts as a positive regulator, which is not indicated. See text and cited references for further discussion of these genes.

three additional synMuv B genes: *lin-9* Aly, *lin-15*B and *lin-36*. LIN-9 is a member of a family of proteins conserved from plants to humans (Beitel *et al.*, 2000; White-Cooper *et al.*, 2000). The *aly* (always early) gene in *Drosophila* encodes a member of this family and has been implicated in transcriptional control of genes required for spermatid differentiation and cell-cycle progression during male gametogenesis (White-Cooper *et al.*, 2000). *lin-15*B and *lin-36* each encode proteins with no obvious orthologs in other organisms. Interestingly, the LIN-36 protein contains an exceptionally high number (19) of potential CDK phosphorylation sites (S/T P) and a conserved cyclin binding domain (ARARLFGE, compare to AKRRLFGE in p107). These properties make it tempting to speculate that LIN-36 is directly regulated by CDKs. Finally, our finding that *lin-15*B acts in parallel to *lin-35* in G_1 regulation provides the first demonstration of a role for *lin-15*B separate from the *lin-35* Rb pathway.

What is the relationship between inhibition of vulval fate specification and inhibition of cell-cycle progression? Regulation of both processes apparently involves transcriptional repression mediated by Rb/E2F complexes. However, the *lin-35* Rb pathway cooperates with distinct regulators to control cell fate specification (class A synMuv genes) and cell division (*cki-1,2* Cip/Kip genes). Moreover, only a subset of the class B genes was found to act in cell-cycle regulation. Therefore, the function of Rb/E2F in cell-fate determination versus cell division control appears to be specified by different sets of coregulators.

Supplementary Material

Supplementary Material including the Experimental Procedures and additional data and discussion is available at http://images.cellpress.com/supmat/supmatin.htm.

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Chapter 5

Genetic screens to identify novel Cyclin D targets and genes that act with *lin-35* Rb during development of *C. elegans*

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ABSTRACT

Taking advantage of the conserved G_1 regulatory mechanisms in *C. elegans*, we have designed forward genetic screens to identify novel genes involved in G_1 control. In a screen for novel *cyd-1* target genes, we identified the *he121* allele. Combining *he121* with loss of *lin-35* Rb restores fertility to *cyd-1* Cyclin D and *cdk-4* CDK4/6 mutants. Therefore, *he121* likely defines a critical downstream effector of *cyd-1* and *cdk-4*. In a second screen, we identified mutations whose phenotype is rescued by loss of *lin-35* Rb. Such mutations may define genes that act upstream of or in parallel to *lin-35* Rb in G_1 control. Finally, we identified a mutation that is viable by itself, but lethal in combination with loss of *lin-35* Rb. This latter type of mutation may help define targets for cancer therapeutics that specifically eliminate cells that lack Rb function.

INTRODUCTION

Regulation of progression through G_1 phase of the cell cycle plays a critical role in the development of multicellular organisms and in the prevention of tumor formation. The decision of a cell to withdraw from the cell cycle or enter a new division cycle is made largely during G_1 phase. Cells become committed to go through a division cycle at a certain point in G_1 phase, after which they are relatively unresponsive to mitogenic and antimitogenic signaling (Pardee, 1989). The importance of G_1 regulation is demonstrated by the fact that most, if not all, tumor cells have sustained defects in one or more G_1 regulators (Sherr, 1996).

At the heart of the molecular machinery that controls progression through G_1 phase stands the retinoblastoma family, consisting of pRb, p107 and p130 in mammals (reviewed in Planas-Silva and Weinberg, 1997; Weinberg, 1995). In order for a cell to progress into S-phase, pRb needs to be inactivated by phosphorylation on several different residues (Mittnacht, 1998; Weinberg, 1995). Inactivation of pRb is brought about by the activities of cyclin dependent kinases (CDKs). Cyclin D-CDK4/6 and Cyclin E-CDK2 phosphorylate pRb on partially overlapping sites, and the activity of both appears to be required for full inactivation of pRb (Mittnacht, 1998). While Cyclin E levels peak shortly before the onset of S phase, the levels of D-type cyclins are much more constant. Cyclin D levels drop when growth factors are withdrawn, and increase when growth factors are added. For this reason, Cyclin D is often thought of as a growth factor sensor (reviewed in Sherr, 1996). One model for G_1 progression is that initial phosphorylation by Cyclin D-dependent kinases inactivates pRb sufficiently to allow transcription of Cyclin E, which then complexes with CDK2 and fully inactivates pRb (Harbour *et al.*, 1999).

The pRb family is currently the only known target for phosphorylation by Cyclin D-dependent kinases. However, Cyclin D-CDK4/6 complexes may fulfill a kinase independent function which involves sequestration of CDK inhibitors of the Cip/Kip family. Members of the Cip/Kip family, which consists of $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$, bind to and inactivate CDK2 kinases. In addition, Cip/Kip family members are essential for the assembly and activity of Cyclin D-CDK4/6 complexes, and most Cyclin D-CDK4/6 is found in complexes with Cip/Kip proteins (Ekholm and Reed, 2000; Sherr, 2000). Thus, Cyclin D-CDK4/6 complexes have been proposed to promote G_1 progression in two ways. First, they can phosphorylate and inactivate pRb and second, they can bind to Cip/Kip kinase inhibitors, and thereby prevent them from inactivating Cyclin E-CDK2.

Two additional functions for Cyclin D have been described that do not depend on association with a CDK partner. First, Cyclin D1 can bind to and activate the estrogen receptor, which might play a role in the formation of breast tumors (Neuman *et al.*, 1997; Zwijsen *et al.*, 1998). Second, Cyclin D1 can bind the Myb-like transcription factor DMP1 (Hirai and Sherr, 1996). Binding of Cyclin D1 to DMP1 is thought to promote G_1 progression by inhibiting transcription of $p19^{ARF}$, which can induce a G_1 arrest by stabilizing p53 (Inoue *et al.*, 1999). The importance *in vivo* for these and other functions of Cyclin D still remains largely unknown. Therefore, we have initiated a genetic approach to identify Cyclin D target genes in *C. elegans*.

The *C. elegans* genome contains single homologs of D-type cyclins, CDK4/CDK6 kinases and pRb family members. We and others have demonstrated that loss of function of either *cyd-1* Cyclin D or *cdk-4* CDK4/6, causes arrest of postembryonic cell divisions in G_1 phase (Boxem and van den Heuvel, 2001; Park and Krause, 1999). Inactivation of *lin-35* Rb in a *cyd-1* or *cdk-4* mutant background partially restores cell division and DNA synthesis. In addition, loss of *lin-35* restores expression of the S phase marker *rnr::GFP* in *cyd-1* and *cdk-4* mutants. These results indicated that *lin-35* is an important negative regulator of G_1 progression and likely acts downstream of *cyd-1/cdk-4* (Boxem and van den Heuvel, 2001). The finding that the rescue of cell division is incomplete indicates that inactivation of *lin-35* Rb is not the only function of *cyd-1/cdk-4*. Here, we describe the identification of a mutation *(he121)* that restores viability to *cyd-1* mutants when combined with inactivation of *lin-35* Rb.

In an alternative approach to identify genes that act to regulate G_1 progression, we performed a genetic modifier screen that is aimed at identifying mutations whose phenotype is either enhanced or suppressed by loss of *lin-35* Rb function. Several different classes of genes can potentially be identified in this screen. First, we can identify genes that act upstream of *lin-35* in G_1 regulation. Thus far, *cyd-1* and *cdk-4* are the only known G_1 regulators that act upstream of *lin-35* in *C. elegans*.

Mutations whose phenotype is suppressed by loss of *lin-35* function may define additional genes that act in the *lin-35* pathway for G_1 control. Second, we may identify novel G_1 regulators that act in parallel to *lin-35* Rb. We have previously demonstrated that loss of *lin-35* affects cell-cycle progression only when combined with mutations in other cell-cycle regulators such as *cyd-1* Cyclin D, *cdk-4* CDK4/6 or *cki-1* Cip/Kip. Therefore, mutations that display a synthetic cell-division phenotype in double mutant combinations with *lin-35* Rb are likely to define genes in the Cip/Kip and other parallel pathways (Fig. 3). A final, and particularly interesting, class of mutations causes a very limited phenotype as single mutants yet results in a lethal phenotype in combination with inactivation of *lin-35*. The gene products defined by such mutations are candidate targets for therapeutics that may kill tumor cells that have lost pRb function, without affecting normal cells.

RESULTS AND DISCUSSION

Identification of a candidate critical *cyd-1* target

To identify novel *cyd-1* target genes we screened for mutations that confer viability to *lin-35; cyd-1* double mutants. Animals of genotype *lin-35(n745); rol-1(e91) cyd-1(he112)/mnC1* were mutagenized with 25 mM ethylmethanesulfonate as described (Brenner, 1974). Individual F1 animals were placed onto 60 mm agar plates, and their F2 progeny was examined for the presence of fertile rolling animals. *lin-35; rol-1 cyd-1* mutants are fully penetrant sterile, despite completing a significant number of postembryonic cell divisions and occasionally producing oocytes. The *mnC1* rearrangement in the parental strain prevents recombination between *rol-1* and *cyd-1*. Therefore, fertile rollers are likely to be homozygous for *cyd-1(he112)* and to contain a mutation that confers fertility to *lin-35; rol-1 cyd-1* mutants. In a screen of ~20,000 haploid genomes we identified three mutations that resulted in viable rolling animals. Two of these mutations were not characterized further as the resulting rolling animals could only be propagated for a few generations. The third mutation, designated *he121*, resulted in healthy rolling animals that produced a high number of viable progeny over several generations. Continuous culture of *lin-35(n745)* mutant animals leads to severely decreased fertility (our unpublished observation). Therefore, *he121* was maintained in a heterozygous strain of genotype *lin-35/dpy-5 unc-29; rol-1 cyd-1/mnC1; he121*.

Initially we considered two trivial explanations for the effects of *he121*. First, we determined if the *he121* mutation represented an intragenic revertant of the *cyd-1(he112)* allele. As such revertants are expected to be dominant, *rol-1(e91) cyd-1(he112)/mnC1; he121* hermaphrodites were crossed to *rol-1(e91) cyd-1(he112)/mnC1* males and placed on agar plates seeded with *E. coli* that express double stranded *lin-35* RNA.

The feeding of double stranded RNA in this fashion has recently been demonstrated to induce RNA mediated interference of gene expression (Kamath *et al.*, 2000; Timmons *et al.*, 2001), and causes efficient inactivation of *lin-35* Rb function (data not shown). If the *he121* mutation reverts *cyd-1(he112)* to a wild type acting *cyd-1* allele, one quarter of the cross progeny would be of the genotype *rol-1(e91) cyd-1(he112 he121)/rol-1(e91) cyd-1(he112)* and display a fertile Rol phenotype. Fertile rollers were not found in the cross progeny, which indicates that *he121* is a recessive mutation, and not simply an intragenic revertant of *cyd-1(he112).*

A second possibility for the rescue of *cyd-1(he112)* by *he121* is that *he121* is an informational suppressor, such as an amber suppressor, or a *smg* suppressor. Amber suppressors are tRNAs that decode UAG stop codons as sense, thus allowing translation to continue past premature UAG codons (Riddle *et al.*, 1997). *smg* mutations affect nonsense-mediated mRNA decay, which is responsible for degradation of mRNAs that contain premature stop codons. Stabilization of such mRNAs by *smg* mutations has been shown to suppress the phenotypes of several mutants (Riddle *et al.*, 1997). The *cyd-1(he112)* allele contains a premature TAG termination codon in the final exon of *cyd-1.* Therefore, *cyd-1(he112)* could in theory be suppressed by an amber or *smg* suppressor. To test this hypothesis we injected *cyd-1* double stranded RNA into *lin-35(n745); rol-1(e91) cyd-1(he112)/mnC1; he121* animals. Whereas wild-type animals injected with *cyd-1* dsRNA produced a high percentage *cyd-1* mutant progeny, *lin-35; rol-1 cyd-1/mnC1; he121* injected with *cyd-1* dsRNA (n=5) produced only fertile rollers. Control injection of *cyb-3* RNA into *lin-35; rol-1 cyd-1/mnC1; he121* animals (n=3) resulted in embryonically arrested progeny, demonstrating that the *lin-35; rol-1 cyd-1/mnC1; he121* strain is not insensitive to RNAi in general. Thus, the *he121* mutation specifically overcomes the *cyd-1(RNAi)* phenotype, which makes it highly unlikely that *he121* is an amber or *smg* suppressor mutation. In addition, we did not observe rescue of the *cyd-1(he112)* mutant phenotype by the *smg-6(r896)* allele among the progeny of *rol-1 cyd-1; smg-6* animals.

Finally, we tested whether *he121* could also overcome the cell division defects of *cdk-4* CDK4/6 mutants. We observed viable progeny from several *lin-35(n745); rol-1(e91) cyd-1(he112)/mnC1; he121* animals injected with dsRNA corresponding to *cdk-4.* In the same experiment, control injected wild-type animals produced a high percentage of larval lethal and sterile progeny. In addition, *lin-35(n745)/+; cdk-4(gv3) rnr::GFP/he121* animals segregated fertile *cdk-4 rnr::GFP* progeny. This result further strengthens the conclusion that *he121* is not an informational suppressor or a genetic revertant of *cyd-1(he112).* Moreover, the fact that *he121* suppresses both the *cyd-1* and *cdk-4* phenotype is consistent with this mutation defining a downstream target of *cyd-1/cdk-4.*

To examine if the rescue of the *cyd-1(he112)* phenotype by *he121* requires simultaneous inactivation of *lin-35*, we placed *rol-1(e91) cyd-1(he112)/mnC1; he121* animals on normal agar plates and on *lin-35* RNAi feeding plates. Fertile rollers were only observed among the progeny on the *lin-35* RNAi plates, demonstrating that inactivation of *lin-35* is required for viability of *cyd-1(he112);he121* double mutants.

Mapping of he121

he121 was mapped to the X chromosome using polymorphic Sequence-Tagged Sites between the N2 Bristol and RW7000 Bergerac chromosomes (Williams *et al.*, 1992). To further define the map position of *he121*, we are making use of single nucleotide polymorphisms between the N2 background and the Hawaiian strain CB4856, which allows for rapid gene mapping in fine detail (Wicks *et al.*, 2001). In this procedure, *rol-1(e91) cyd-1(he112)/mnC1; dpy-6(e14) he121* animals are mated with the CB4856 strain and the cross-progeny is allowed to self-fertilize on *lin-35* RNAi feeding plates. From the F2 animals, fertile rolling non-Dpy animals are selected and used to establish homozygous Rol non-Dpy strains. Such recombinant strains are either of genotype *rol-1(e91) II; CB4856 X* or *rol-1(e91) cyd-1(he112) II; he121 CB4856 X*. To select the latter strains we remove homozygous animals from the *lin-35* RNAi plates. Their progeny should display the *cyd-1* phenotype, as rescue requires both *lin-35* inactivation and the *he121* mutation. Recombinant *rol-1(e91) cyd-1(he112) II; he121 CB4856 X* strains will be analyzed for the presence of the CB4856 or N2 sequence at multiple single nucleotide polymorphisms along the X chromosome.

Functional characterization of he121

As a first step in the functional characterization of the gene defined by *he121*, we examined whether *he121* alone was able to rescue aspects of the *cyd-1* mutant phenotype. *cyd-1; he121* double mutants showed a sig-

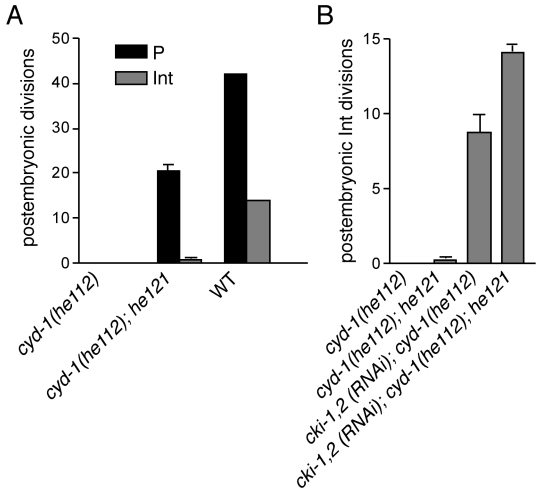

Fig. 1. (A) The *he121* allele partially restores cell divisions in *cyd-1(he112)* mutants. Postembryonic intestinal (Int) and P neuroblast (P) divisions were determined for 10 animals of the indicated genotypes. (B) *he121* may cooperate with *cki-1,2* in negatively regulating G_1 progression. Postembryonic intestinal divisions were determined for 20 animals of indicated genotypes. Intestinal nuclei were marked with *elt-2::GFP*. Bars represent mean ± s.e.m.

nificant number of postembryonic cell divisions of, among other cell types, the ventral cord precursor (P) cells (Fig. 1A) and the hypodermal (V) cells (not shown). Divisions of the intestinal nuclei were barely affected by *he121* (Fig. 1) and endoreduplication did not take place in the intestinal nuclei of *cyd-1; he121* mutants (not shown). These results further demonstrate that the gene defined by *he121* likely acts downstream of *cyd-1*.

As described in the introduction, Cyclin D-CDK4/6 complexes are thought to affect progression through G_1 phase by two distinct mechanisms: phosphorylation of pRb and sequestration of Cip/Kip kinase inhibitors. Both of these mechanisms may be conserved in *C. elegans* as loss of *lin-35* Rb or loss of *cki-1,2* Cip/Kip partially rescues the *cyd-1* and *cdk-4* mutant phenotype (Boxem and van den Heuvel, 2001). The gene defined by *he121* might act in a *cki-1,2* Cip/Kip pathway, or may be part of a third, parallel pathway (Fig. 2). However, *he121* is not an allele of *cki-1* or *cki-2*, as both these genes map to chromosome II. To address if *he121* acts in parallel to *cki-1,2*, we examined the effect of double inactivation of *cki-1,2* and *he121* on intestinal divisions (Fig. 1B). Combining *cki-1,2* RNAi and the *he121* allele resulted in a higher number of intestinal divisions in *cyd-1* mutants than *cki-1,2* RNAi or the *he121* allele alone. This may indicate that *he121* acts in parallel to *cki-1,2*. However, a synergistic effect may be also be obtained by combining partial loss-of-function of two genes that act in the same pathway. At present, we do not know whether *he121* is a null allele or whether *cki-1,2* RNAi completely eliminates *cki-1,2* function. Therefore, it remains possible that *he121* acts in the *cki-1,2* Cip/Kip pathway.

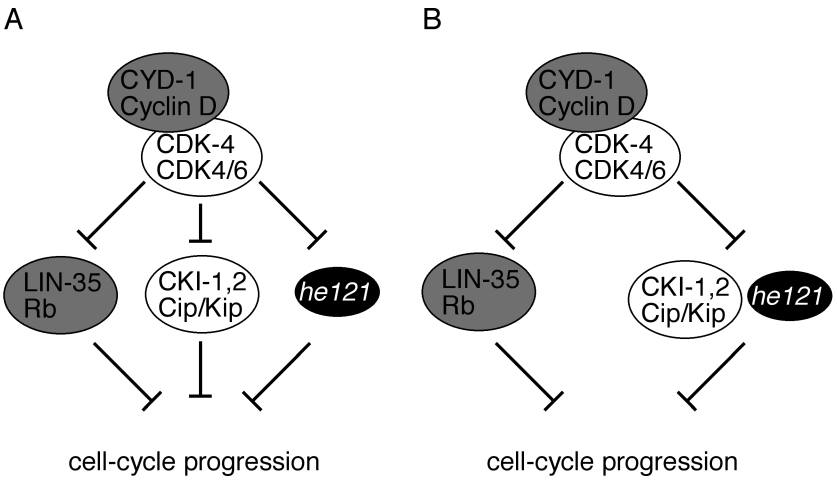

Fig. 2. Model for the function of *he121*. LIN-35 and CKI-1,2 act downstream of CYD-1/CDK-4 in two parallel pathways. *he121* may act downstream of CYD-1/CDK-4 in a third pathway (A), or, alternatively, may act upstream or downstream of CKI-1,2 (B). *he121* cannot be a mutant allele of *cki*-1 or *cki*-2, as both *cki-1* and *cki-2* are located on chromosome II, while *he121* was mapped to the X chromosome.

In summary, we have identified a highly interesting mutation, *he121*. This mutation likely defines a gene that acts in parallel to *lin-35* Rb, and needs to be downregulated by *cyd-1/cdk-4* for the development of *C. elegans*. The molecular characterization of this gene will help to elucidate which Cyclin D-CDK4/6 targets are essential for the development of multicellular organisms.

A screen for the identification of genes that cooperate with *lin-35* Rb during development of *C. elegans*

As outlined in the introduction and Fig. 3, mutations that genetically interact with *lin-35* loss of function may help define genes in several important developmental pathways. To identify such mutations, wild type animals were mutagenized with 25 mM ethylmethanesulfonate as described (Brenner, 1974). Single F2 progeny, which are potentially homozygous for the induced mutations, were allowed to produce self-progeny for 24 hours and subsequently transferred to *lin-35* RNAi feeding plates. The first plates were kept at 15°C while the *lin-35* RNAi feeding plates were kept at 25°C. After four days, the normal and *lin-35* RNAi plates were compared and strains that displayed a developmental abnormality on one plate but not the other were selected for further examination. Because of the temperature difference between the normal and RNAi plates, many strains were discarded early on as their phenotype was simply due to a temperature sensitive mutation and not to the presence or absence of *lin-35*. In future screens all plates will be maintained at the same temperature to avoid this problem. Also note that due to the nature of the screen we could not identify fully penetrant lethal or sterile mutations that were only partly suppressed by loss

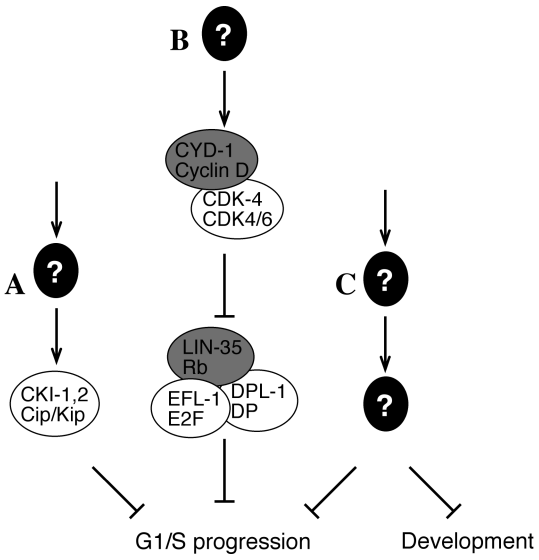

Fig. 3. A screen for mutations that genetically interact with *lin-35* Rb may define genes in multiple important pathways. Mutations that demonstrate a synthetic interaction with loss of *lin-35* Rb may define genes that act in parallel to *lin-35* Rb, either in the *cki-1,2* Cip/Kip pathway (A) or in other pathways (C). In addition, mutations whose phenotype is suppressed by *lin-35* Rb loss of function may define genes that act upstream of *lin-35* Rb (B).

of *lin-35*, as F2 animals homozygous for such mutations would not produce progeny.

In an initial screen of approximately 1225 haploid genomes we identified 10 candidate mutations. In addition, three class A synMuv alleles were found, which further validates the screen. Nine of the 10 mutations actually represent mutations whose phenotype is rescued by loss of *lin-35*. Two classes among these are maternal effect embryonic lethal mutations that survive to fertile adults on *lin-35* RNAi plates (2/10) and partially penetrant sterile mutations that are nearly wild type on *lin-35* RNAi plates (3/10). One of the mutatant strains displayed a more severe phenotype on *lin-35* RNAi plates. Animals of this strain were largely fertile on normal plates and fully penetrant sterile when placed on *lin-35* RNAi feeding plates. As this screen has the potential to isolate several important classes of developmental regulators (Fig. 3) and has already been shown to be successful, it will be carried out at a comprehensive level.

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GENERAL DISCUSSION

In this thesis, I describe experiments in which *C. elegans* was used as an animal model to study developmental control of cell divisions. The results we obtained contributed to our understanding of the basic cell-cycle machinery in *C. elegans*, and demonstrated that the functions of key cell-cycle regulators are conserved between *C. elegans* and mammals. This indicates that further research into the regulation of cell division in *C. elegans* will translate into improved knowledge of the mammalian cell cycle. In addition, the findings on the basic cell-cycle machinery enabled us to design genetic screens to identify novel regulators of G_1 progression. In a reverse genetic approach, we identified several novel negative regulators of G_1 progression. In forward genetic screens we identified a mutation that likely defines a critical target for Cyclin D-CDK4/6 kinases, and several mutations that may define genes that cooperate with the Rb family during development of *C. elegans*. Below, I briefly discuss our results and the contributions they made to our understanding of the mammalian cell cycle.

Similar mechanisms regulate cell division in C. elegans *and mammals*

Traditionally, the regulation of the cell cycle received little attention in *C. elegans* and few cell-cycle genes were previously described for this organism. Therefore, our goal was to identify and characterize components of the core cell-cycle machinery in *C. elegans*. We demonstrated that *ncc-1* CDK1 is required specifically for entry into mitosis and progression through meiosis (Boxem *et al.*, 1999). In addition, an ortholog of CDK4/CDK6 kinases (*cdk-4*), and a D-type cyclin (*cyd-1*) are required for G_1 progression of postembryonic somatic cells (Boxem and van den Heuvel, 2001; Park and Krause, 1999). Furthermore, a homolog of the mammalian Cip/Kip kinase inhibitors, *cki-1*, was shown to negatively regulate cell-cycle progression (Boxem and van den Heuvel, 2001; Feng *et al.*, 1999; Hong *et al.*, 1998). Finally, we determined the role of *lin-35* Rb, *efl-1* E2F and *dpl-1* DP in G_1 progression and found that *lin-35* and *efl-1* are negative regulators of G_1 progression while *dpl-1* has aspects of both a positive regulator and a negative regulator. In each case, the *C. elegans* cell-cycle gene acts in a similar fashion as its mammalian counterpart. This establishes *C. elegans* as an attractive genetic system in which to study cell-cycle regulation, as results obtained in *C. elegans* will likely be applicable to cell-cycle regulation in mammals.

The functions of Cyclin D-CDK4/6 kinases in vivo

Mammalian Cyclin D-dependent kinase complexes are thought to act in at least two ways. First, they can phosphorylate and inactivate pRb family members (Mittnacht, 1998; Sherr, 1993). Second, they can sequester members of the Cip/Kip family of CDK inhibitors, which inhibit CDK2 kinases (Sherr and Roberts, 1999). To date, no experiments have directly determined the relative importance of these mechanisms for the function of D-type cyclins and CDK4/6 kinases *in vivo*. Inactivation of *lin-35* Rb, the single *C. elegans* Rb-related gene, significantly rescued the cell division defects in *cyd-1* Cyclin D and *cdk-4* CDK4/6 mutants. Thus, in *C. elegans*, inactivation of LIN-35 Rb appears to be one critical function of the CYD-1/CDK4 complex. This is the first demonstration that inactivation of pRb family members is an important function of Cyclin D-CDK4/6 kinases *in vivo*.

As the rescue of the *cyd-1* Cyclin D mutant phenotype by loss of *lin-35* Rb is not complete, other *cyd-1* targets likely exist. Indeed, in a forward genetic screen we identified a mutation that rescues *lin-35* Rb*; cyd-1* Cyclin D double mutants to fertile adults. Although sequestration of Cip/Kip proteins is a possible second function of Cyclin D-CDK4/6 kinases, this mutation is not an allele of the *C. elegans* Cip/Kip family members *cki-1* or *cki-2*. Identifying the gene affected by this mutation should help define the nature of the second function of *cyd-1* Cyclin D.

lin-35 *Rb acts primarily in a negative regulatory complex with* efl-1 *E2F*

An important question about the function of mammalian pRb family members is to what extent the E2F transcription factors mediate their function. Mammalian E2Fs likely act both as transcriptional activators and, in a complex with pRb family members, as transcriptional repressors. It is currently unknown which of these two mechanisms is most relevant for the function of E2F *in vivo*. In addition, it is unknown to what extent pRb is recruited to the DNA through factors other than E2F. We found that loss of *efl-1* E2F rescued the cell division defects of *cyd-1* Cyclin D mutants to a similar extent as loss of *lin-35* Rb. Therefore, most of the G_1 inhibitory function of *lin-35* Rb appears to be mediated by *efl-1* E2F. Ceol *et al.* (Ceol and Horvitz) recently demonstrated the possibility of a ternary LIN-35/EFL-1/DPL-1 complex. LIN-35 Rb is, therefore, likely part of an inhibitory complex containing EFL-1 and DPL-1.

Using C. elegans *to isolate novel cell-cycle regulators*

The major strength of studies in *C. elegans* is the ability to use genetic techniques to identify novel genes. We performed several screens to identify novel regulators of G_1 progression. *lin-35* Rb, *efl-1* E2F and *dpl-1* DP each regulate G_1 progression as well as vulval cell fate specification (Ceol and Horvitz, 2001; Ferguson and Horvitz, 1985; Ferguson *et al.*, 1987 and Chapter 4). Using a reverse genetic approach, we identified a role for 3 additional synMuv genes (*lin-9* Aly*, lin-15*B and *lin-36*) as negative regulators of G_1 progression. These genes all encode proteins for

which no function in G_1 regulation had previously been described. *lin-15*B and *lin-36* both encode novel proteins. *lin-9* is a member of a family of proteins conserved from plants to humans. A *Drosophila* homolog of *lin-9*, *always early* (*aly*), is required for the transcription of genes required for spermatid differentiation and the transition from G_2 to meiosis during the formation of male gametes (White-Cooper *et al.*, 2000). Both LIN-9 and LIN-36 contain an exceptionally large number of candidate CDK phosphorylation sites, indicating that these proteins may be directly regulated by CDKs.

In Chapter 5 I described forward genetic screens to identify novel targets of *cyd-1* and genes that cooperate with *lin-35* Rb during development. One of the identified mutations likely defines a gene that acts downstream of *cyd-1*. In addition, we have already identified mutations whose phenotype is suppressed by loss of *lin-35*, and mutations that are lethal only when combined with inactivation of *lin-35*. I expect that such mutations define several classes of genes. First, similar to *cyd-1* and *cdk-4*, mutations whose phenotype is suppressed by loss of *lin-35* may define G_1 regulators acting upstream of *lin-35*. Second, mutations whose phenotype is enhanced by loss of *lin-35* may define genes that act in parallel to *lin-35*, similar to *cki-1* and the synMuv class A genes. Finally, mutations that are viable by themselves but lethal in combination with loss of *lin-35* may ultimately define genes that are candidate targets for therapeutics that specifically kill tumor cells that lack pRb. These results show that these screens have great potential to identify novel cell-cycle regulators.

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Mike

CURRICULUM VITAE

Ik ben geboren op 23 maart 1974 in Lichtenvoorde. In 1992 behaalde ik mijn V.W.O. diploma aan het Veenlanden College in Mijdrecht. In datzelfde jaar begon ik met de studie Medische Biologie, aan de Vrije Universiteit te Amsterdam. In het 1995 liep ik stage op de afdeling Orale Microbiologie van de Vrije Universiteit, onder begeleiding van Dr. Peter Willemsen. In 1996 liep ik stage bij de afdeling Celbiologie van het Nederlands Kanker Instituut, in de groep van John Collard, onder begeleiding van Dr. Peter Hordijk. In 1997 liep ik een derde stage in de groep van Dr. Sander van den Heuvel aan het Massachusetts General Hospital Cancer Center in de Verenigde Staten. In mei van dat jaar rondde ik mijn studie af, en begon ik met mijn promotieonderzoek in de groep van Dr. Van den Heuvel. Daar bestudeerde ik de regulatie van celdelingen in de nematode *Caenorhabditis elegans*. Gedurende mijn promotieonderzoek gaf ik voordrachten over mijn werk tijdens onder andere de 2000 Eastcoast Worm Meeting (Atlanta), en de 2001 Internationale Worm Meeting (Los Angeles). Van januari 2000 tot juni 2002 ontving ik een predoctorale beurs van het Boehringer Ingelheim Fonds.

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